JOURNAL

OF

BACTERIOLOGY

VOLUME XXV

BALTIMORE, MD.
1933

Medical
Wm. H. W.

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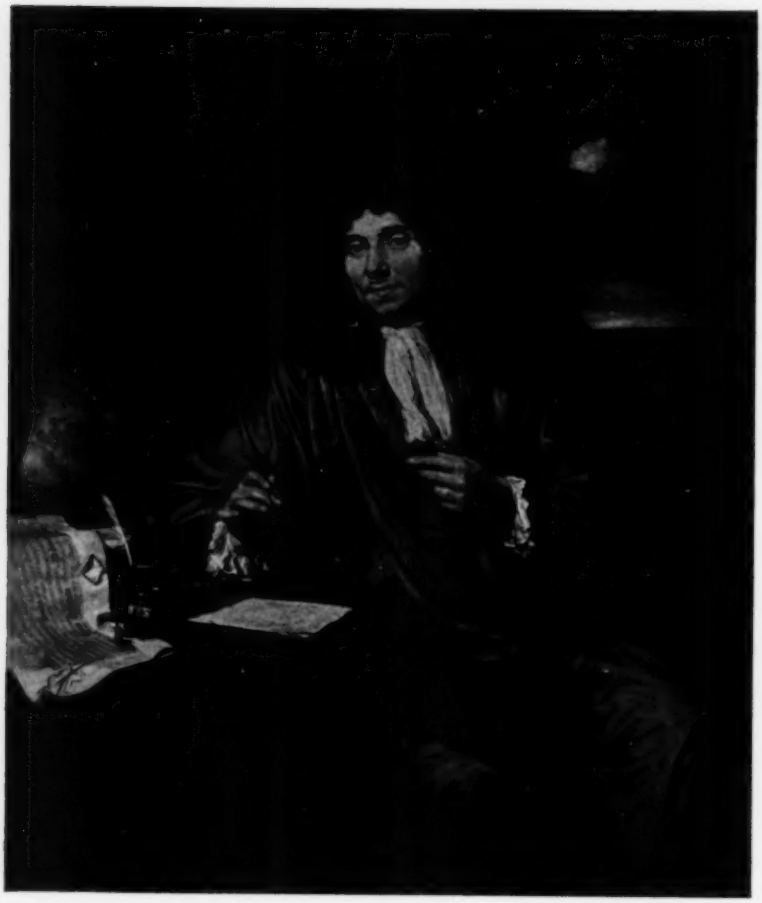

ANTONY van LEEUWENHOEK
1632-1723

ANTONY VAN LEEUWENHOEK[1]

ON THE THREE-HUNDREDTH ANNIVERSARY OF HIS BIRTH

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This year the scientific world everywhere is celebrating the anniversary of the birth of Antony van Leeuwenhoek. Just three hundred years ago in the fair and prosperous city of Delft, Holland, Antony, son of Philips Antonysz van Leeuwenhoek, was born. Like many another celebrity, young Leeuwenhoek sprang from a sturdy and honorable family of simple tastes and limited means. His plain way of living and love for nature may possibly be attributed in a measure to his parentage, but his remarkable power of seeing the hitherto unseen in the world of microscopic life cannot in any way be explained by his heredity. The Leeuwenhoek family has been traced back for a few generations but no record of remarkable skill has been discovered.

It takes many generations to evaluate a great man, and often his followers receive the praise while the original genius is almost forgotten. In a way this is true of Leeuwenhoek. His brilliant followers, of almost two centuries later, Pasteur, Koch, Lister, and others are well known for their important discoveries in the microscopic world first opened up by Leeuwenhoek.

Leeuwenhoek, the Polymath of Microbiology, exhibited marvelous technique as well as keen power of observation in his studies of protozoa, bacteria and other forms of life. No one, either before or since Leeuwenhoek, working with such simple microscopes has so clearly and accurately described the forms which we call the microörganisms. Leeuwenhoek saw so many of these small forms and told so accurately of their mode of life

[1] Presidential Address delivered before the Society of American Bacteriologists at its Thirty-fourth Annual Meeting, Ann Arbor, Michigan, December 29, 1932.

that we are amazed at his keen perception and almost unbelievable power of unlocking the secrets of nature. One may well imagine his excitement when he first beheld these microscopic forms. It is true that he did not try to connect the relation of these animalcules to the applied field; his rôle was that of "creator of tiny things;" and in this rôle he stands out today as the pioneer of microbiology.

The world owes much to Leeuwenhoek. Although many years passed before anyone truly appreciated the importance of his discoveries, it is clearly established that he laid the foundation for the great subject of bacteriology. From the small beginning of 1676, when Leeuwenhoek first saw bacteria in rain water, an entirely new science has developed, a science of microbiology which to-day is throwing out ramifications into numerous fields. Fate has linked the thirty-fourth annual meeting of the Society of American Bacteriologists with the tercentenary of the birth of the Father of Bacteriology. Of these three centuries, a scant seventy years are all that have been devoted to the serious application of a scientific study of the bacteria.

In the preparation of this paper, the author has drawn freely from a remarkable book entitled, "Antony van Leeuwenhoek and His 'Little Animals'," by Clifford Dobell. This English protozoölogist possesses an unrivalled knowledge of the Leeuwenhoek manuscripts and it is indeed fortunate that he has seen fit to publish the results of his exhaustive studies.

EARLY LIFE

Leeuwenhoek was born on October 24, 1632. His father as well as his grandfather were craftsmen, simple basket-makers, and his mother the daughter of a Delft brewer. In his family there were seven children, five girls and two boys. Only one of the girls, Catharina by name, is mentioned in connection with her brother's scientific achievements. About the personal life of young Leeuwenhoek we know but little; his father died when he was only five years old, and his early years were spent at Warmond, a small town near Leyden. Later we find him at Benthuizen, living with his uncle. At the age of sixteen he entered the shop

of a linen-draper in Amsterdam, where he no doubt became acquainted with the crude magnifying lens used to examine threads in cloth. Perhaps this simple microscope showed the beautiful patterns of threads interlaced in the warp and woof of the cloth. This introduction to the microscopic world no doubt stimulated his imagination to go beyond the thread counter and to delve into mysteries as yet unheard of. At any rate, Leeuwenhoek began to grind better lenses and by means of these lenses to see more of the invisible world. Nothing is known of the next five or six years except that when he was about twenty-two, Leeuwenhoek returned to Delft and married Barbara de Mey. This was, so far as we know, a happy marriage, of which there were five children, though only one, named Maria, survived him. About this time (1654), Leeuwenhoek bought a house and set up a clothing store in Delft. Six years later he received an appointment as Chamberlain to the Sheriffs of Delft, a position which he held for thirty-nine years and thereafter until his death received the salary of this office. At first this position paid only 314 florins each year, but later 400 florins. Fortunately, as was the custom of that time the position was largely a sinecure and thus Leeuwenhoek was free to devote his life to the grinding of lenses and the focusing of his microscopes on almost every object which came within his reach.

That Leeuwenhoek was interested in mathematics and had more than a speaking acquaintance with this subject is indicated in his letters and is recorded in the Dutch Archives. In the year 1669 Leeuwenhoek was examined by the mathematician, Genesius Baen, and found competent to perform the duties of surveyor (Schierbeek, 1930). The fact that he was qualified as a surveyor no doubt explains the mathematical calculations found in certain of his letters. Just ten years later in 1679, Leeuwenhoek was elected wine-gauger for the city of Delft. These positions, surveyor and wine-gauger, required the attention of a well-trained man and thus is seems hardly conceivable that he should be classed as an ordinary beadle as he has been by some of his biographers. Leeuwenhoek was in a very real sense a man of affairs in his native city of Delft.

HOLLAND IN THE SEVENTEENTH CENTURY

To gain some idea of our man and his work, we must read his original letters and those of his associates and consider, too, conditions in Holland during his life. The seventeenth century included the reign of Frederick Henry (1625–1647), the Golden Age of Holland. At this time, the affairs of Holland flourished, an eastern empire was acquired, and conquests were made in South America. It was a time when religion and politics were not always uppermost in the mind of man, certainly not in Leeuwenhoek's mind, a period when learning, literature, and painting reached a dazzling brilliancy.

The Seventeenth Century is well known as the period of illustrious scientists not only in Holland but also in other countries. The roster of scientists for this era carries the names of such men as Robert Boyle, Christopher Wren, Robert Hooke, Nehemiah Grew, Gottfried Leibniz, Marcello Malpighi, Francesco Redi, Jan Swammerdam, John Ray, William Harvey, Christiaan Huygens, Isaac Newton, and others of lesser fame. From this grand array of geniuses and super-geniuses, it seems that Leeuwenhoek received but little, either by direct contact or by reading. He could not read Latin or any other language but his mother tongue. He never attended a scientific meeting, and never had any contact with a university. In a very real sense of the word he was a lone worker, though in later life he met and exchanged papers with certain of his contemporaries. Some idea of how he disliked advice from visitors is indicated in his letter to Oldenburg (1675).

> Your Excellency recommends me to make use of the services of other people, who are in a position to form a proper judgment of such things. Sir, I must say that there be few persons in this Town from whom I can get any help; and among those who can come to visit me from abroad, I have just lately had one who was much rather inclined to deck himself out with my feathers, than to offer me a helping hand.

THE LEEUWENHOEK MICROSCOPES

The first magnifying glasses were produced by spectacle-makers. At first only a low magnification (about 10 fold) was employed.

Although the compound microscope was described by Zacharias Janssen of Middelburg as early as 1590 to 1610, it was never used by Leeuwenhoek. The simple, small, biconvex lens prepared from glass, or in a few cases from rock-crystal, mounted between two plates usually of brass, silver, or in rare cases, of gold, and fastened by little rivets was the form of microscope commonly employed by Leeuwenhoek. From the various descriptions of his instruments it seems that he also made use of two or three lenses mounted in the same frame. It should be borne in mind that Leeuwenhoek was a glass-blower of no mean ability. Coupled with this skill in working glass he showed rare ingenuity and expert craftsmanship in the grinding and mounting of his simple lenses, a skill which he zealously kept to himself; and in spite of the requests of his learned friends, he refused to disclose the secret of his success. He was a self-taught man and naturally jealous of his own inventions; hence it is understandable that he would not give away the details of his method of making microscopes and of their use.

To adjust the lens to the object was so long and tedious a task that it is not surprising that Leeuwenhoek used an individual lens for each object and fixed this object in place by gluing it on the point of a pin attached to the microscope. Leeuwenhoek made many microscopes; the exact number is not known, although he left 247 not only with lenses but usually with objects in place. He also left 172 plates with one lens in each, or a total of 419 lenses.

A brief description of the microscopes by Leeuwenhoek, himself, is found in the extract of his letter of the 2nd of August, 1701, published by Weld (1848).

Hon^{ble} Gentlemen:

My last to your honours was dated the 21st June, wherein I humbly offer'd you my observations about spiders, since when I have received the book which treats of fishes, and the whole set of Philosophical Transactions for the year 1700, for which noble presents I return you my most hearty thanks.

I have a small black cabinet, lacker'd and gilded, which has five little drawers in it, wherein are contained thirteen long and square tin boxes,

covered with black leather. In each of these boxes are two ground microscopes, in all six and twenty; which I did grind myself, and set in silver; and most of the silver was what I had extracted from minerals, and separated from the gold that was mixed with it; and an account of each glass goes along with them.[2]

This cabinet, with the aforesaid microscopes (which I shall make use of as long as I live), I have directed my only daughter to send to your honors, as soon as I am dead, as a mark of my gratitude, and acknowledgement of the great honor which I have received from the Royal Society.

This account in Leeuwenhoek's own words gives some idea of the methods used in making these early microscopes.

A description and in some cases diagrams of Leeuwenhoek's lenses will be found in the papers of Folkes (1724), Baker (1753), Harting (1859), Disney (1928), and Dobell (1932). According to these accounts, Leeuwenhoek's instruments are not true microscopes at all, in the sense in which we think of microscopes, but rather simple magnifying glasses generally consisting of a small single biconvex lens. The object, and not the lens, was moved into focus by means of screws.

The magnification varied and at best did not exceed 200 to 300 diameters. How it was possible to see such small objects as bacteria with such a low magnification, and without the use of dyes remains a matter of speculation. Perhaps the solution is to be found in the fact that Leeuwenhoek possessed rare ability in the grinding of his lenses, as well as in the preparation and mounting of his objects. To remove any doubt of the possibility of his having seen such small forms with a lens of low power, Chapman (1931) has imitated the conditions of the Leeuwenhoek experiment. He mounted the front lens of a 1/12-inch objective in much the same way as Leeuwenhoek and thus obtained a magnification of 125 diameters. After a little practice with this type of lens Chapman found that he could see yeast cells very clearly and also bacteria. Dobell (1932) has advanced a quite

[2] These microscopes were exhibited to Peter the Great, when he visited Delft in 1698. The Czar requested Leeuwenhoek to pay him a visit on his canal yacht outside the town of Delft, and to bring some of his admirable microscopes, adding, that he would have gone to visit him at his residence, had it not been for the wish he had to escape the notice of the multitude.

different explanation to account for the remarkable clarity of Leeuwenhoek's observations. He suggests that Leeuwenhoek made use of some form of dark-ground illumination, for in a letter of 1675 Leeuwenhoek drew an analogy to the appearance of sand grains on "black taffety silk." Although we do not have any record of how Leeuwenhoek carried out his microscopic observations it seems probable that his remarkable discoveries represent simply the reward of a keen eye coupled with unparalleled industry, rather than any revolutionary change in technique. According to Hooke, Leeuwenhoek used a capillary pipette in the examination of liquids.

The size of objects which Leeuwenhoek examined was determined by comparison. For this purpose he used at various times a grain of sand, the seed of millet or mustard, the eye of a louse, a vinegar eel, and still later hair or blood corpuscles. In this way he secured fairly accurate measurements of a great variety of objects.

During his lifetime Leeuwenhoek refused to sell any of his microscopes and it was not until many years after his death that a collection of his instruments was put up for sale. Harting tells of the auction sale of these microscopes and of the fact that the gold microscopes of Leeuwenhoek were sold according to weight. Perhaps no other microscopes were ever sold on such a basis. Unfortunately, at present there are only 8 authentic specimens of Leeuwenhoek's microscopes in existence (Dobell, 1932).

LEEUWENHOEK'S DISCOVERIES IN BACTERIOLOGY

It was not until he was 41 years old that Leeuwenhoek began to impart to the world the various things which were within the range of vision of his simple microscope. Fortunately, there lived in Delft at that time (1673) the famous Dr. Reinier de Graaf, a young physician of rare ability best known for his description of the "Graafian follicle," who recognized the value of Leeuwenhoek's work and persuaded him to send his reports to the scientific group in London. Thus through the influence of de Graaf who was corresponding with Oldenburg, the Secretary of the Royal Society, the early letters of Leeuwenhoek were sent to this

scientific body. During the same year, Constantijn Huygens also sent to the Royal Society a letter describing the qualifications of Leeuwenhoek. The value of these letters was soon recognized and in 1680, Leeuwenhoek was elected a Fellow of the Royal Society, an honor which he prized highly. Leeuwenhoek's fondness for the smallest forms of life, the "little animals," as he called them, is recorded in many of his letters. This love of the small unicellular forms of nature kept up throughout his long life of ninety-one years. His observations were submitted in the form of letters, usually to the Royal Society of London, or to some of his friends. On his very death bed we find him calling for his friend, Jan Hoogvliet, to translate two letters to send to the Royal Society. No attempt will be made here to deal fully with the variety of things which Leeuwenhoek described, but simply to give certain examples of his discoveries relating to the bacteria, yeasts and moulds.

The following description of the mouldy growth on the surface of meat is taken from Leeuwenhoek's first letter to the Royal Society, London, April 28, 1673. Unfortunately this letter is not a fair example of Leeuwenhoek's work. The description is rather crude.

A specimen of some observations made by a microscope, contrived by M. Leeuwenhoeck in Holland, lately communicated by Dr. Regnerus de Graaf, written from Delpht, April 28, 1673.

The mould upon skin, flesh, or other things, hath been by some represented to be shott out in the form of the stalks of vegetables, so as that some of those stalks appeared with round knobs at the end, some with blossom-like leaves. But I do observe such mould to shoot up first with a straight transparent stalk, in which stalk is driven up a globous substance, which for the most part places itself at the top of the stalk, and is follow'd by another globul, driving out the first either sideways, or at the top, and that is succeeded by a third and more such globuls; all which make up at last one great knob on the stalk, an hundred times thicker than the stalk itself. And this knob indeed consists of nothing else than of many small roundish knobs, which being multiplied, the big knob begins to burst asunder, and then represents a kind of blossoms with leaves.

Leeuwenhoek discovered yeasts in fermenting beer and wine as recorded in his letter of June 14, 1680: ". . . . these seem to be quite round; others were irregular and seemed to consist of two, three or four of the aforesaid particles joined together."

The discovery of bacteria is given in Leeuwenhoek's celebrated letter of October 9, 1676, to the Royal Society. In this communication, he mentions that one year earlier he found living creatures in rain water which had been stored for a few days in a new tub. At this time he described bacteria and various kinds of animalcules of which the majority were undoubtedly protozoa. He said:

The 2nd observation. Rain-water (After Dobell)

The 26th of May, it rained very hard. The rain abating somewhat, I took a clean glass and got rain-water that came off a slate roof, fetched me in it, after the glass had first been swilled out two or three times with rain-water. I then examined it, and therein discovered some few very little animals; . . . .

In the same report he attempted an estimate of the size of these little creatures, saying they (perhaps as Dobell suggests *Monas vulgaris*) were to a cheese-mite in the same proportion as a honey Bee to a Horse.

Apart from protozoa, he found exceedingly small animalcules, " . . . . they well-nigh escaped the sight" in stored rain water, well and sea water. To satisfy his curiosity about the burning effect of pepper, he prepared a pepper infusion. This he allowed to stand, and after about three weeks he found in this infusion five sorts of animalcules, of which the fourth and fifth sorts were bacteria, probably bacilli. This account as given by Dobell follows:

The fourth sort of little animals, which drifted among the three sorts aforesaid, were incredibly small; nay so small, in my sight, that I judged that even if 100 of these very wee animals lay stretched out one against another, they could not reach to the length of a grain of coarse sand; and if this be true, then ten hundred thousand of these living creatures could scarce equal the bulk of a coarse sand-grain.

In a letter of March 23, 1677, he observed that water which had been boiled would, when kept for 24 hours, show the presence of living creatures.

In his famous letter of September 17, 1683, concerning scrapings from teeth and again in 1693, he gave a description of the different forms of bacteria. His own account follows:

> Tho my Teeth are kept usually very clean, nevertheless when I view them in a Magnifying Glass, I find growing between them a little white matter as thick as wetted flower: in this substance tho I could not perceive any motion, I judged there might probably be living Creatures.
>
> I therefore took some of this flower and mixt it either with pure rain water wherein were no Animals; or else with some of my Spittle (having no Air bubbles to cause a motion in it) and then to my great surprize perceived that the aforesaid matter contained very many small living Animals, which moved themselves very extravagantly. The biggest sort had the shape of A. (reference to a figure) their motion was strong and nimble, and they darted themselves thro the water or spittle, as a Jack or Pike does thro the water. These were generally not many in number. The 2d. sort had the shape of B. these spun about like a top, and took a course sometimes on one side, as is shown at G. and D. they were more in number than the first. In the 3d sort I could not well distinguish the Figure, for sometimes it seem'd to be an Oval, and other times a Circle. These were so small they seem'd no bigger than E. and therewithal so swift, that I can compare them to nothing better than a swarm of Flies or Gnats, flying and turning among one another in a small space.

Later in this same report he says:

> I took in my mouth some very strong wine-Vinegar, and closing my Teeth, I gargled and rinsed them very well with the Vinegar, afterwards I washed them very well with fair water, but there were an innumerable quantity of Animals yet remaining in the scurf upon the Teeth, yet most in that between the teeth, and very few Animals of the first sort A.
>
> I took a very little wine-Vinegar and mixt it with the water in which the scurf was dissolved, whereupon the Animals dyed presently. From hence I conclude, that the Vinegar with which I washt my Teeth, kill'd only those Animals which were on the outside of the scurf, but did not pass thro the whole substance of it.

Returning to his letter of June 14, 1680, we find not only a description of the yeasts, but also of the anaerobic bacteria. Leeuwenhoek had apparently heard it said that life could not exist in a closed vessel and with this in mind he sealed a glass tube containing an infusion of pepper. Five days later, he broke this seal and found the infusion contained various kinds of bacteria.

Another example from a letter of M. Antony Leeuwenhoek, dated from Delft, November 4, 1681, is given below:

Containing an account of several new Discoveries made by him this last Summer.

I have also several times this Summer walked into our Meadows, in order to observe the fresh Excrements of Cows, Horses, and other Animals, but I could not yet discover any Animals in them: but I found multitudes of these small Globules swimming in a clear liquor, some of which were not above one sixth part, others not above a thirty sixth part of a blood Globule.

In urine of a mare he found,

. . . . a great variety of differing Globules, some as big as those of blood, and composed of six others: these first were like a very close bunch of Grapes, whence though they were not perfectly round, yet I call them Globules; amongst these I found some 1/6 of a Blood Globule, ...

In his homely way, Leeuwenhoek says the "Animals in the scurf of a man's Teeth are so many that I believe they exceed the number of Men in a kingdom."

In addition to his study of the occurrence of these microörganisms it must be remembered that Leeuwenhoek also began the first work on the physiology of the bacteria. The effect of heat, hot coffee, of acid, of tobacco on the bacteria were included in his experiments. Indeed, we might well say that Leeuwenhoek laid the foundation for our well-known process of pasteurization. His early experiments in which he observed the harmful effect of heat on bacteria support this conclusion. In addition Leeuwenhoek also was the first to prepare a nutrient medium for bacteria, *e.g.*, his infusions of pepper and other condiments. These and

many other important facts relating to the physiology of the microorganisms are to be found in the letters of this savant from Delft.

During his long and productive life, Leeuwenhoek sent no less than 200 letters to the Royal Society of London. Usually each letter tells of some new discovery, often in an entirely new field. As reported by Dobell the signatures of these letters are not uniform: the first 39 were signed *Antony Leeuwenhoeck* and the later reports after 1683 *Antonj van Leeuwenhoek*, the *c* from -hoeck dropped and the *van* added. All told, the various English versions of his letters published in the Philosophical Transactions of the Royal Society show 19 different ways of spelling the surname.

His work, as it appears in the letters, stands out in the form of isolated pieces of information rather than as organized knowledge concerning the nature of microscopic forms of life. The method of publishing his letters in chronological order has accentuated this point. If these same letters are rearranged according to subject matter, seemingly unrelated facts fall into a natural sequence, and thus the underlying thought becomes apparent.

It is indeed fortunate that these original manuscripts in the records of the Royal Society are well perserved. The ink has faded but little, the drawings are in certain cases excellent, the hand writing is clear and fairly easy to read.

THE TWENTY-SEVEN LOST LETTERS

The Nederlandsch Tijdschrift voor Geneeskunde (*Dutch Journal of Medicine*) at Amsterdam (1931) has recently published the 14 (not 27) letters of Leeuwenhoek which have not appeared before in print. During the years that these 14 letters have remained undiscovered in the archives of the Royal Society, there have grown up many wild speculations. It now appears that there is nothing mysterious about these letters. The story as taken from Dobell is somewhat as follows: The Dutch and Latin editions of Leeuwenhoek's works begin with the 28th letter. Of the 27 it has been found that 13 have already been published in the whole or in part and hence only 14 remain. These 14 letters are now reproduced in their original language and are also translated into English. In the main these 14 letters do not add anything very

startling. They represent the early work of Leeuwenhoek which was revised and in the main presented again in his later reports.

SPONTANEOUS GENERATION

Leeuwenhoek was not content simply to note the presence of the "animalcules" but also tried to find out the source of these forms of life. That they could not originate by spontaneous generation was clear in Leeuwenhoek's mind although such a conception was quite at variance with that of many of his contemporaries, notably Jean van Helmont. Leeuwenhoek vigorously opposed the idea of spontaneous generation as noted in his letters of October 9, 1676 and of May 13, 1680. To explain the presence of the "animalcules" in pure rain water or in rain water sealed in glass tubes he suggested that the seeds of these minute organisms can exist in water which is drawn up into the clouds and later scattered in the form of rain. Unfortunately, he did not attempt to extend his observations of this very important phenomenon, and thus almost 100 to 150 years elapsed before the idea of spontaneous generation was shown to be false.

NATURE OF LEEUWENHOEK'S WORK

The question we may well ask is how it was possible for one man to cover such a wide range of subjects. The answer is not easy. His ability to "estimate what was worth doing and what could be done" shows that Leeuwenhoek possessed a creative mind of the highest order. In the words of Chapman, 1931, Leeuwenhoek possessed an "infinite capacity for taking pains which may be defined as genius." He was fortunate in many ways: no time-consuming administrative work fell to his lot; no popular subject of the day demanded his attention; he loved his microscopes, and that love grew stronger as the years went by. Unmindful of the world of big things, he was satisfied to keep ever prying into nature's secrets. He did not attempt to seek the universal elixir or to accomplish the transmutation of the metals, which were the popular fads of his day.

The homely and simple language used in his letters to the Royal Society give us some idea of his unique power of observation. A

paragraph from Hoole's, "The Select Works of Antony van Leeuwenhoek," 1816, will bring out these points.

> I have often heard, that many persons dispute the truth of what I advance in my writings, saying that my narrations concerning animalcules, or minute living creatures, are merely of my own invention. And, it seems, some persons in France have even ventured to assert, that those are not in truth living creatures, which I describe as discoverable to our sight, and alledge, that after water has been boiled, those particles in it which I pronounce to be animalcules will be still observed to move. The contrary of this, however, I have demonstrated to many eminent men, and I will be bold to say, that those gentlemen who hold this language, have not attained to a degree of proficiency to observe such objects truly. For my own part, I will not scruple to assert, that I can clearly place before my eye the smallest species of those animalcules concerning which I now write, and can as plainly see them endued with life, as with the naked eye we behold small flies, or gnats sporting in the open air, though these animalcules are more than a million of degrees less than a large grain of sand. For I not only behold their motions in all directions, but I also see them turn about, remain still, and sometimes expire; and the larger kinds of them I as plainly perceive running along, as we do mice with the naked eye.

It has been said that scientific curiosity is "the divine instinct." If this be true, then surely Leeuwenhoek should receive the mantle of Elijah; for probably no one has ever had to a greater degree that insatiable desire to inquire into the processes of the microscopic world.

Leeuwenhoek was not without honor during his lifetime. Scientists and nobility came to Delft to see this man of the microscopic world. Peter the Great of Russia, Queen Mary of William and Mary, Frederick I of Prussia, and others stopped in Delft to call on Leeuwenhoek. Like the public of to-day, these rulers of the seventeenth century were impressed with the scientific developments of their day. They too were curious to know what lies in the world beyond the unaided eye of man.

MICROBIOLOGY BEFORE AND AFTER LEEUWENHOEK

The influence of Leeuwenhoek's findings upon the discoveries of subsequent workers is not easy to estimate. All that is modern

in microbiology has been influenced either directly or indirectly by his observations. During his long life time, he examined the minute animals, and the bacteria in water from rain, from melting snow, from wells, from canals and from rivers. Indeed, wherever plant or animal substances were decomposing, he found various kinds of bacteria. The originality and amazing insight of the man is well exemplified by the breadth of his discoveries.

Of the immense number of objects examined, we shall speak only of his studies of the bacteria. Leeuwenhoek was not content merely to see the bacteria in water which had been kept for days and also in scrapings from the teeth, but he also studied the forms in pepper infusion which was kept in a closed bottle. Here he found anaerobic forms, probably the butyric acid bacteria. These results naturally led him to examine the sap of plants, soil, urine and manure where he also found great numbers of microörganisms. He observed the chief morphological types, the cocci, bacilli and spirilla.

From the life of this wonderful man of Holland there are many lessons we all may learn. There are to-day, no doubt, many works of nature still waiting for the keen eye and patient mind of a second Leeuwenhoek. Unaided by association with the best minds of his day he rose to stand in first place among the world's great microbiologists. His observations are examples of a self-trained scientist. He was endowed with rare mechanical skill, with insatiable curiosity, unparalleled patience and exquisite power of observation. With these qualifications, it seems certain that if he had worked at a later date he would have been quick to recognize the difference in behavior of microörganisms, as well as the important rôle they play in health and disease. As shown in a letter to Robert Boyle, 1677, he was a modest, unselfish man. The numerous honors conferred upon him, the visits of royalty from various nations, did not influence his simple tastes. How beautifully his own words written towards the close of his career express his feelings towards science and scientists (Dobell translation).

As I aim at nothing but Truth, and, so far as in me lieth, to point out Mistakes that may have crept into certain Matters; I hope that in so

doing those I chance to censure will not take it ill: and if they would expose any Errors in my own Discoveries, I'd esteem it a Service; all the more, because 'twould thereby give me encouragement towards the attaining of a nicer Accuracy.

There is another characteristic of Leeuwenhoek we must mention: he possessed the art of turning his observations into a tale free from the formal stilted language of the usual scientific memoir. The original letters must be read to appreciate his simple, sincere style and conversational way of expressing his discoveries. His biographer, Dobell, has very aptly described this style of Leeuwenhoeck as typified by his letters. "Generally they are long and rambling and his thoughts come tumbling out with disconcerting inconsequence." "He never confuses his facts with his speculations."

It seems appropriate in closing to give the inscription from the silver medal presented to Leeuwenhoek by the University of Louvain which so beautifully describes the work of the First Microbiologist:

In tenui labor, at tenuis non gloria

His work was with small things but his glory is not small

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SCIENTIFIC PROCEEDINGS

THIRTY-FOURTH ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

LEEUWENHOEK TERCENTENNIAL

ANN ARBOR, MICHIGAN, DECEMBER 28, 29 AND 30, 1932

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ABSTRACTS*

Report of Committee on Bacteriological Technic: Progress during 1932.
H. J. Conn, *Chairman*; Victor Burke, Ivan C. Hall, J. A. Kennedy, Barnett Cohen, Elizabeth F. Genung, *Members.*

A year ago this Committee reported that the revision of the *Manual of Methods for Pure Culture Study of Bacteria* had been finished but that one leaflet was still lacking to complete the entire series contemplated. This missing section has now been published as Leaflet VII on *The Study of Pathogenic Aerobes*, by Victor Burke, Committeeman on Pathological Methods. This was issued in August and has since been included in all copies of the Manual sold. Besides this addition, Leaflets III, IV, VI and VIII have been revised during the year. As a result, the Manual is now very different from what it was as recently as two years ago.

The new leaflets for the Manual have always been sent to the subscribers of the Continuation Service. It was announced a year ago that this Continuation Service was being changed into the form of a quarterly journal. Publication of this journal, under the name of *Pure Culture Study of Bacteria*, began in February and it has been subsequently issued in May, August and November. It is in these four numbers of the journal that the above mentioned leaflets to the Manual have been published. Notification of the change into periodical form was sent to all subscribers of the Continuation Service and most of them have renewed their subscriptions as they ran out. We have now, accordingly, 575 subscriptions to the new journal. Although this is a smaller number than we reported a year ago as on the Continuation Service list, there is good reason to believe that quite a number of the lapsed subscriptions will be renewed as soon as the subscribers fully understand the change.

Up to the current year the Committee has turned into the Society exactly 25 per cent of all receipts on the sale of charts, Manuals, and subscriptions to the Continuation Service. This has amounted to around $300 a year. This year, however, on account of the additional expense of starting the new journal, together with the increased number of pages added to the Manual, it has been necessary to depart from this policy temporarily. Twenty-five per cent of the receipts from the sale of the Descriptive Charts and Manuals is being turned into the Society's

* This number of the Journal has been edited by the Chairman of the Program Committee. Authors of the abstracts in the Scientific Proceedings have not seen proof, owing to restrictions of time imposed by the printing schedule.

treasury, but any similar payment on the new journal is being postponed until another year.

The Chairman of the Committee still acts as Chairman of the Commission on Standardization of Biological Stains. The Committee thus assists in the testing of all stains put on the market in this country. As reported last year, the appropriations from the Chemical Foundation have been decreased. A further cut has been taken this year, and more difficulty has been experienced, therefore, in financing the work. It has been continued without curtailment, however, and requests for financial assistance from individuals or from scientific organizations have not yet been necessary.

GENERAL BACTERIOLOGY

G1. Rationalization, from the Viewpoint of Physical Chemistry, of the Behavior of Bacteria Toward Dyes, with Special Reference to Staining. ALLEN E. STEARN, University of Missouri, Columbia, Mo.

The concept of the bacterial cell as an equilibrated system of two classes of components, the one distinctly more acidic than the other, which, as a system, is also amphoteric, is useful in correlating its behavior toward chemical agents, especially dyes. Such a general view is justified by the following considerations:

1. Simple synthetic systems of the above kind are found to be amphoteric, and differ from pure ampholytes in that they show great buffering power at their isoelectric points in place of the small or vanishing buffering power shown in general by pure simple ampholytes. Data available on the buffering power of bacteria show them to buffer well at their isoelectric points (Falk and Shaughnessy, 1922–23).

2. Bacteria are known to be amphoteric not only by their chemical behavior but also by their electrokinetic behavior (Winslow, et al., 1923 to 1926). Studies on the electrokinetic behavior yield results predictable as characteristic of an amphoteric system of more than one component with mutual combination between the components at the isoelectric point. Thus the isoelectric point of the system as a whole will lie between the respective isoelectric points of the two classes of components, and within this range the one may act as a base and the other as an acid. Behavior toward dyes also indicates varying degrees of mutual combination of the components at the isoelectric point of the organism in that certain organisms tend to retain *both* acid and basic dyes at this point while others retain neither.

On the alkaline side of their isoelectric points bacteria retain basic dyes with increasing tenacity as the alkalinity is increased, while on the acid side they are increasingly easily freed from such dyes by decolorizer but tend increasingly to retain acid dyes. The reaction between bacterial constituents and dyes seems to follow the laws of chemical stoichiometry. In fact certain easily available cell constituents (e.g., yeast nucleic acid) which there is no reason to think essentially different from bacterial constituents, can be easily titrated to a definite and sharp endpoint, under proper conditions, by means of a solution of such a dye as crystal violet. Dye specificity is governed by the relative dissociations of the dye-bacterial salts in a manner analogous to well known ionic displacements.

The isoelectric points of the bacteria are roughly characteristic of the various types. Thus, if a group of different organisms be treated with a staining solution, those with isoelectric points of lowest pH will tend to retain the dye most strongly if it is a basic dye. This inherent difference in dye retention could be used as the basis of a crude classification. Actual methods employ the refinements of mordants and standardized decolorizing procedures. The Gram classification has as its basis the above inherent differences in bacterial types. The so-called mordant, usually Lugol's iodine, effects an amplification of the inherent differences in the isoelectric points of the two groups.

The important action of the mordant, at least in this case, is on the bacterium rather than on the stain. Recent work has shown that Gram-positive organisms bind more iodine than do Gram-negative, and that this difference is not affected by pretreatment with gentian violet (Habs, 1932). Of less importance are such factors as the effect of salts on the appearance of dye solutions, as for example the fact that iodides change gentian violet solutions from a violet color to a pure deep blue.

At best there will be some organisms with isoelectric points of such a value that only a highly standardized technic will give consistent results. The isoelectric points of bacterial types are not absolutely fixed. Even in the analogous synthetic systems they vary with the relative concentrations of the two constituents, so that in bacteria they may be expected to vary somewhat with the conditions of growth, primarily the type of medium. For this reason certain organisms will be legitimately found to be Gram-variable, while others are consistently positive or negative.

In any dye technic the type of decolorizer is of utmost importance. In the ordinary Gram technic, if the ethyl alcohol be replaced by the

more acidic methyl alcohol, a somewhat different classification would result. (Note that this is not a pH effect.) More organisms would be pushed into the Gram-negative group. On the other hand if the less acidic amyl or octyl alcohol were used, more organisms would be pushed into the Gram-positive group.

The importance of a rough regulation of pH, which has been empirically introduced into modifications of the Gram technic, finds its theoretical justification and explanation also on this general view.

There seems to be no essential difference between the mechanism of dyes as stains and as bacteriostatic agents. Organisms show isoelectric behavior to dye bacteriostats. They are increasingly sensitive to basic dyes as the pH is increased, and to acid dyes as it is decreased; a culture actually completely inhibited in growth for a long period of time by a basic dye may be caused to grow out by decreasing the pH, holding all other factors constant; organisms inherently staining most strongly are in general most sensitive to the staining dye, and it has been demonstrated, at least in some cases, that a change in sensitivity to dye in the case of a variant is accompanied by a corresponding change in the isoelectric point.

Finally it may be pointed out that, empirically, bacteria show amphoteric properties toward the flocculating action of acid and basic dyes. At present, the analogy with the staining and stasis behavior, together with the general theory of colloidal behavior, is the only basis for suggesting a mechanism for such flocculating behavior.

G2. The Relation between the Bacteriostatic Action of Gentian Violet and the Oxidation-Reduction Potential of the Medium. MARY A. INGRAHAM AND E. B. FRED, University of Wisconsin, Madison.

The recent work of Stearn and Stearn which indicates that the bacteriostatic action of a dye such as gentian violet is due to the formation of a dye-protein complex, and depends on the basicity of the dye ion and on the isoelectric point of the cell, does not entirely explain the striking changes in bacteriostatic power which accompany changes in the size of the inoculum. A correlation between the dye-tolerance of many species, and their reducing ability, suggested the following studies.

Preliminary work on growth curves showed that non-lethal doses of gentian violet prolong the lag period, but have little effect on either the subsequent rate of growth or upon the fermentation products. A practical method for measuring this effect on the lag was developed, which allowed the logarithm of the number of cells in the inoculum to

be plotted against the time required to produce turbidity in a liquid medium. The resulting curve is a straight line, the slope of which changes with the concentration of the dye. The ratio of the slope of the line representing growth without dye, to that of the line representing growth with dye is an index to the sensitivity of the species tested in a given medium.

Just as there is no change in fermentation products, provided logarithmic growth is once initiated, so there is no difference in the oxidation-reduction potentials developed with and without gentian violet.

Potentiometric data, however, indicate that gentian violet acts as a poising agent in many bacteriological media. The addition of a given amount of an oxidizing or reducing agent has appreciably less effect on the oxidation-reduction potential in the presence of dye concentrations used in bacteriostatic work, than in their absence. At optimum potentials for growth, the action of the dye is least marked, other conditions being constant, but at unfavorable potentials increased bacterial action is required before the oxidation-reduction potential is brought to a range permitting the initiation of logarithmic growth. The inhibitive action of oxidizing or reducing agents may be partially modified by the addition of suitable concentrations of gentian violet, clearly showing that the same mechanism is affected by both. Other poising agents such as ferric ammonium sulphate differentiate between tolerant and sensitive species, as does gentian violet. It is therefore concluded that the bacteriostatic action of gentian violet is a function of its poising capacity.

G3. Surface Properties of Intestinal Bacteria Before and After Serum Sensitization. ELEANORE W. JOFFE, CHARLES H. HITCHCOCK AND STUART MUDD, Department of Bacteriology, School of Medicine, University of Pennsylvania.

Data concerning the surface properties of certain strains of intestinal bacteria are presented. A non-flagellate typhoid strain, 0901, a Flexner dysentery strain, and a strain of *Bact. enteritidis* have been studied in their smooth and rough variants. The enteritidis bacilli were grown on phenol agar to suppress the flagella. The smooth variants carry surface electric charges so low as to be scarcely measurable, but form even suspensions, insensitive to precipitation by salt. The rough variants grow as a sediment and their suspensions are salt-sensitive, but, paradoxically, they have high surface charges.

Sensitization of these intestinal bacteria with specific immune sera gives them cataphoretic properties presumably approaching those of

the antibody protein. Sensitization with rabbit antisera has given the maximally sensitized bacteria isoelectric points between pH 4.8 and 5.2; with horse antisera the isoelectric points have been between pH 4.4 and 4.8. These isoelectric points are well on the acid side of those of normal serum globulins. These results suggest that the antibody globulins against bacteria of this group may be more acid than normal globulins rather than more alkaline like those previously described.

G4. The Stimulation of Bacterial Metabolism by Bacteriophage. J. BRONFENBRENNER, Washington University Medical School, St. Louis, Mo.

In following the gross changes taking place in the cultures of susceptible bacteria in the presence of bacteriophage, we observed the increase in the rate of bacterial growth (as expressed either by the turbidity of the culture as a whole or by the rate of division of bacteria as recorded by the cinematographic camera) taking place very early after the introduction of bacteriophage and continuing up to the time when the lysis of bacteria begins. A careful observation of the progress of lysis under the microscope indicated that the disappearance of individual bacteria under the influence of bacteriophage is usually preceded by more or less marked swelling of the cells. A more detailed inquiry indicated that the swelling itself was due to imbibition of water, which in turn appeared to be the result of intracellular digestion of bacterial cytoplasm, and consequent increase of osmotic pressure within the cells. The actual disappearance of cells from the field was therefore interpreted as due to bursting.

While the above observations were made with gram-positive as well as gram-negative bacteria, the changes were always more constant and more marked in the case of the latter and therefore the gram-negative bacteria were used to illustrate the phenomenon. Recently, there appeared in the literature several papers in which, mostly on the basis of somewhat indirect evidence, authors concluded that, at least in the case of staphylococcus cultures, lysis proceeds without the preliminary phase of accelerated growth and without the swelling of cells.

This paper reports the results of an attempt to reinvestigate the question. Experiments designed to test the existence of the stimulation were performed taking advantage of the fact that bacteria upon which bacteriophage is adsorbed fail to undergo lysis when transferred upon media with high concentration of gelatin or agar. This procedure per-

mits making count of bacteria actually existing in the culture at any moment, and the counts thus obtained were compared with the counts from the culture in which the same bacteria were grown in the absence of bacteriophage.

The results thus secured indicate that the growth of bacteria in the cultures containing bacteriophage goes on at an increased rate. The same conclusion was drawn from another set of experiments in which droplets with bacteriophage were deposited upon the surface of agar on which an even growth of bacteria was secured beforehand. On the plate containing low concentration of agar the deposition of bacteriophage resulted in the formation of minute plaques of lysis. However, when media with high agar content are substituted and similar procedure is followed it will be found that instead of lysis, under each drop of bacteriophage there appears a greatly increased growth of bacteria. The "klatch" preparations taken from such plates indicate that in all cases in which lysis has taken place, bacteria at the edge of the plaque are invariably swollen, whereas bacteria taken from the plate containing high concentration of agar and showing increase in growth rate remain normal in size. These experiments were performed using staphylococcus, *B. megatherium* and *B. diphtheriae* and thus leave no doubt that phenomena described previously by us in connection with the lysis of *B. coli* can be reproduced also with the gram-positive bacteria. These results suggest in general that the primary effect of bacteriophage consists in excitation of metabolic activity of bacteria. The lysis is a secondary phenomenon, independent of bacteriophage and governed by the osmotic changes in the bacterial cells.

G5. The Stimulating Effect of Potato Extract on the Butyric Acid Bacteria.
E. L. Tatum and W. H. Peterson, University of Wisconsin, Madison.

The addition of water extract of potato to corn mash brought about a vigorous fermentation of the mash by certain butyric acid bacteria. In the presence of the extract the fermentation was characterized by an extensive gas production, great turbidity of the supernatant medium, "head" formation and almost complete destruction of starch. This increased starch destruction resulted in a markedly increased production of butyl alcohol. The amounts of other solvents and acids remained practically unchanged. The degree of stimulation caused by potato extract varied greatly with the strain of butyric acid organism used, although a certain stimulation was observed with all the strains tried.

Butyl Beijerinck No. 21 was most pronouncedly stimulated. The stimulating factor was found to be present in varying amounts in Irish and sweet potatoes, but was lacking in corn, oats, rice and semi-synthetic media containing glucose or starch.

Some of the properties of the active principle are: stable to heating and drying; destroyed by ashing; soluble in water and in 80 per cent alcohol; insoluble in ether, acetone, and in 95 per cent and absolute alcohol; dialyzable through viscose and collodion membranes; not adsorbed on norit, silica-gel, or kaolin; not precipitated by phosphotungstic acid, barium hydroxide or by ammoniacal lead acetate. It was, however, carried down in the precipitation of barium sulfate in the presence of ammonium acetate, and could be recovered from this precipitate by treatment with ammonium hydroxide. The final purification resulted in a preparation of which 9.75 mgm. (dry basis) brought about a vigorous fermentation in 100 cc. of 4 per cent corn mash. This stimulating substance may therefore be classed provisionally as an accessory growth factor.

G6. (*a*) *The Significance of the "Induction Effect" in Mitogenetic Radiation.* Otto Rahn and J. B. Tuthill, Laboratory of Bacteriology, Cornell University, Ithaca, N. Y.

Baron demonstrated mitogenetic radiation by exposing yeast cells to the supposedly radiating tissue. If the percentage of buds, which is usually counted about 2 to 3 hours after exposure, on dried and stained smears, is considerably greater than the percentage in the unexposed controls, mitogenetic radiation is considered established. Several sources of error enter here. The deviation on different parts of the same agar plate was found to amount to 5.8 per cent of the lowest value, or to ± 5 per cent of the average. Some of the buds of *Sacch. ellipsoideus* were found to break off in the preparation of the smear before staining, thus decreasing the percentage.

The percentage of buds is no measure of the growth *rate*. It indicates the growth *phase* of the culture, and the Baron technique measures the rapidity with which the yeast culture comes out of the lag phase.

The computation of the "induction effect" as the percentage increase in buds of the exposed over the unexposed culture is of no definite meaning. From the newer methods of counting cell numbers, the growth rate, or generation time, of test and control can be calculated, and the ratio of these is a true measure of the intensity of radiation upon the growth rate.

G6. (*b*) *Mitogenetic Radiation as the Cause of the Influence of the Number of Organisms upon the Lag Period.* Otto Rahn and Alice Jean Ferguson, Cornell University, Ithaca, N. Y.

Old bacteria brought into a fresh medium do not grow at once at the fastest rate possible in that medium, but require a certain time for "adjustment." This time, called the lag period, depends, among other factors, upon the number of organisms transferred. It decreases as the inoculum becomes larger.

This rather surprising fact has been explained by Baron as being due to mutual irradiation of organisms. According to Gurwitsch, the stimulus of "mitogenetic radiation" is necessary for cell division. The chance of being irradiated increases with the amount of the inoculum. Baron proved his case by absorbing the radiation with gelatin, and thus preventing growth acceleration.

The authors could demonstrate that the lag period of *Bact. coli* was shortened if the culture was placed in a quartz test tube surrounded by a yeast suspension. The radiation from this suspension was strong enough to pass through the quartz and stimulate growth. This agrees with Baron's explanation for the decrease of lag by large inocula.

G6 (*c*) *On the Lethal Radiation from the Human Body.* Otto Rahn and Margaret N. Barnes, Cornell University, Ithaca, N. Y.

The old "superstition" that menstruating women cause flowers to wilt and fermentations to become abnormal, has been verified experimentally by several authors. In 1929, Christiansen proved that menstrual blood will kill yeast cells, or change them morphologically, even through a quartz coverglass.

The authors observed that this influence is not limited to menstruating women. One woman could not obtain growth of a very sensitive species of yeast for periods of 10 to 20 days, alternating with periods of normal growth. Radiation from her fingertips through a quartz plate killed the yeast in 15 minutes. One man killed yeast by this same method in half of all tests. Even his eye and nose, at very close range through quartz, had the same effect. Since this radiation does not pass through glass slides, it is probably ultra-violet.

Observations for more than one year suggest that this radiation is due to pathological conditions. Both the above cases can be explained in this way, and there are other similar observations.

Macht and Lubin have shown the menstrual toxin to be identical

with, or closely related to oxycholesterol. We observed this compound to kill yeast through quartz. So did the ether washings from the fingers of menstruating persons. This kind of radiation may therefore be explained simply by a pathological excretion of oxycholesterol through the skin.

G7. The Oxidation-Reduction Character of Agar Media and the Growth of Aerobic Bacteria. L. W. BROWN AND I. L. BALDWIN, University of Wisconsin, Madison.

The influence of the oxidation-reduction character of agar media on the growth of several common aerobic organisms has been studied by various methods.

The oxidation-reduction level of agar slants has been varied by the addition of peroxide and of such reducing agents as sodium thioglycollate, sodium thiosulfate, and sodium hydrosulfite. The growth of many of the common laboratory aerobes is markedly influenced by such oxidation or reduction of the substrate, particularly by oxidation. Growth is inhibited in most media by small amounts of peroxide, and by moderate concentrations of reducing agents. In many cases the growth of the so-called strictly aerobic organisms is aided by the addition of a mild reducing agent with high capacity, such as thiosulfate. This is particularly true when the medium is one with a high oxidation-reduction potential (e.g., mannitol nitrate). Small concentrations of the stronger reducing agents (thioglycollate or hydrosulfite) may also be beneficial, but the use of sufficient concentrations of these to give reasonable poising or capacity produces a potential level which is too low for the optimum growth of aerobic organisms.

The addition of small amounts of peroxide or permanganate to agar shake-cultures of the "strict" aerobes causes the formation of subsurface zones of growth, or of diffused growth throughout the medium. The depth of such growth depends on the potential and capacity of the untreated medium, as well as upon the amount of oxidizing agent added.

Potentiometric studies have been made by the insertion of electrodes at various distances below the surface of the agar in shake-culture tubes. The potential changes at the various depths, due to the reducing power of the medium and the slow penetration of atmospheric oxygen, correlate well with the color changes of methylene blue in such media. Such potential measurements offer an explanation for the appearance and position of growth zones in shake-cultures.

G8. The Growth of Bacillus megatherium in Relation to Free Oxygen and the Oxidation-Reduction Potential of the Medium. GEORGES KNAYSI AND S. R. DUTKY, Department of Dairy Industry, Cornell University, Ithaca, N. Y.

Bacillus megatherium grows very well in meat infusion broth of pH 6.8 at atmospheric pressure. The potential of that medium, when sterile, gives a reading of about 0.000 volt with reference to the saturated calomel electrode. Under 15 mm. of air pressure at 30°C., growth is very slight. The sterile medium under the latter condition has a potential of about −0.185 v. The critical air pressure for the growth of the organism seems to be about 10 mm. at 30°C. When sodium sulfite is added to meat infusion broth of pH 6.8, a concentration of 0.27 per cent or more inhibits growth. The potential of the medium containing 0.27 per cent of sodium sulfite is about −0.215 v. in the air and does not change on evacuation to 15 mm. of air pressure. The sulfite medium was allowed to stay in the air 24 hours before readings were taken.

B. megatherium grows very well at atmospheric pressure in meat extract broth containing 0.5 per cent of ferric ammonium citrate. The potential is about −0.210 v. and does not change more than a few millivolts on evacuation to an air pressure of 3 mm. at 24°C. However, the organism is completely inhibited under the latter condition.

B. megatherium grows well at atmospheric pressure in Dox's solution, with sodium nitrate as a source of nitrogen, in the presence of 0.1 per cent of ferric ammonium citrate. The potential of this synthetic medium is about +0.090 v. Under 3 mm. of air pressure at 30°C., the potential does not drop below +0.040 v. and yet growth is inhibited.

The free oxygen content of the above media was determined on 20 cc. portions at 30°C. with the following results:

Medium	Free oxygen content cc.
Meat infusion broth, pH 7.0	0.07
Meat infusion broth +0.27 per cent sodium sulfite	0.00
Meat extract broth, pH 7.0	0.10
Meat extract broth +0.5 per cent ferric ammonium citrate	0.15
Dox's solution +0.1 per cent ferric ammonium citrate	0.18

The results here reported show that *B. megatherium* can grow in media of low potentials if free oxygen be present and is inhibited in media of high potentials in the absence of free oxygen. The organism is inhibited in the presence of highly reducing substances, but it is shown that, under

these conditions, free oxygen is absent. One is, therefore, led to conclude that it is the lack of free oxygen, and not the low potential of the medium, that limits the growth of *B. megatherium* in vacuum.

G9. A Study of the Bactericidal, Bacteriolytic or Inhibitory Substance Produced by Some Molds and Some Factors Which Influence Its Production. ROGER D. REID, The Pennsylvania State College.

A group of 30 molds was studied to determine whether or not the production of an inhibitory substance, present in the broth filtrate after culturing *Penicillium rubrum* (Biorge), as reported by Fleming was general among molds. None of the Aspergillus or Mucor varieties studied gave evidence of the ability to produce any of the properties looked for, i.e., bactericidal, bacteriolytic or inhibitory. Of those molds belonging to the group known as the Penicillia, *Penicillium rubrum* (Biorge), *P. rubens*, *P. notatum* (Westling) and *P. chrysogenum*, all closely related forms, showed more or less ability to produce a substance which inhibited *Staphylococcus aureus*, which became the test organism, and some other bacterial forms.

Certain media stimulate the production of the substance. Double strength veal infusion broth was used to cultivate the molds. There is always an increase in the pH of the medium as the inhibitory property increases. The property is most potent at pH 8.0. In testing for the inhibitive property, a large number of bacteria was used. Some were readily inhibited by the use of small quantities of the broth filtrate upon which the mold had been grown. Others were not affected even by large quantities. No groups of bacteria were found in which the inhibition is complete for all species. Organisms which were definitely inhibited did not show clumping when treated with the filtrate.

The substance inhibited the growth of *Corynebacterium diphtheriae* in vitro but produced no protective action and did not cause any local irritation at the site of injection in guinea pigs. The inhibitory factor is non-dialyzable through a collodion membrane. It is non-toxic to animals either upon administration superficially or by injection intraperitoneally.

Although it resists heating at 60° to 90°C. for short periods of time, it begins to lose its inhibitory property at the end of 3 weeks if it is stored at any temperature, ranging from ice-box to 37.5°C. This suggests that heat is not an active factor in the disintegration or loss of the inhibitory property.

G10. Three Contested Types of Bacterial Growth in a Single Species, Bacillus polygenesis. (With motion pictures.) JEAN BROADHURST, Teachers College, Columbia University, New York.

This series of motion pictures shows (1) increase in volume in spreading masses of *Bacillus polygenesis* while entirely lacking in evidences of cell structure and the final formation of distinct cells; (2) the development of giant coccoid cells or "balloon bodies;" and (3) several stages in the development of fungi-like zygotes.

G11. The Utilization of Certain Substituted Sugars by Bacteria. STEWART A. KOSER AND FELIX SAUNDERS, University of Chicago.

In this investigation we have used a number of substituted sugars and have compared the ability of various bacteria to make use of these modified sugar molecules with the utilization of the common sugars from which they were derived.

It was found that the substitution of a methyl group for the hydrogen of the hydroxyl group attached to the number one carbon atom rendered the sugar molecule distinctly less vulnerable to bacterial attack. Thus *Bact. coli*, members of the paratyphoid group, *Bact. typhosum*, *Bact. dysenteriae* Flexner, *Bact. dysenteriae* Sonne, *Staphylococcus aureus* and *albus*, *Corynebacterium diphtheriae* and a yeast were unable to make use of alpha methyl glucoside although all of these organisms fermented glucose readily. A few species, however, were able to ferment the methyl glucoside: *Proteus*, *Bact. friedländeri*, *Bact. aerogenes*, *Diplococcus pneumoniae*, types I, II and III, and streptococci from scarlet fever, erysipelas and septic sore throat.

Similar comparative tests with mannose and alpha methyl mannoside showed that although mannose was used readily by most of the types studied, the methyl mannoside was not fermented by any of the organisms, even those which were able to attack methyl glucoside. In a similar manner, beta methyl *l*-arabinoside and beta methyl *d*-xyloside were not used by those organisms capable of fermenting *l*-arabinose and *d*-xylose or by organisms fermenting lactose which has a beta linkage.

Two sugars containing seven carbon atoms in the chain, alpha glucoheptose and alpha glucoheptulose, were not fermented by any of the organisms. Glucosamine was used by most of the cultures capable of splitting glucose, though in some instances apparently with more difficulty. Gluconic acid was used by many of the bacteria although fermentation tests with the streptococci, pneumococci and two species of yeasts were negative. A sulphur-containing sugar, glucose ethyl mercaptal, gave entirely negative results.

G12. Notes on the Trimethylene Glycol Fermentation. C. H. WERKMAN, C. W. DAVIS AND C. A. TARNUTZER, Department of Bacteriology, Iowa State College, Ames.

Organisms of the genus Citrobacter are characterized as members of the Escherichia-Aerobacter group, citrate positive, M.R. positive, V.-P. negative. To date 75 cultures belonging to this group isolated from soil, water, spoiled eggs, and various animal feces have been studied culturally in detail and found to produce trimethylene glycol from glycerol in a medium in which ammonium chloride was the sole source of nitrogen.

Braak proposes to account for the trimethylene glycol as a reduction product of glycerol functioning as a hydrogen acceptor. The presence of suitable hydrogen acceptors should be reflected in the reduced yield of trimethylene glycol. Experimentally, the addition of fumaric acid prevented the formation of the glycol and resulted in the formation of succinic acid.

The addition of $NaHSO_3$ fixed relatively large quantities of acetaldehyde showing it to be probably an intermediate product; and led to a greatly reduced yield of acetic acid. The addition of the bisulfite prevented the production of trimethylene glycol, a fact which would not favor the suggestion that trimethylene glycol is formed as a direct hydrogenation product of glycerol (Braak, 1928) but rather that an intermediate aldehydic compound is first formed such as hydracrylic aldehyde as suggested by Voisenet, or propionic aldehyde as suggested by Braak in a personal communication.

Aeration also inhibited the formation of trimethylene glycol probably as a result of the behavior of oxygen as a hydrogen acceptor.

The highest yields of trimethylene glycol were very near to one-third by weight of the glycerol fermented (32.1, 32.4, 35.4, 32.0 per cent) seemingly indicating that one molecule of glycerol out of three acts as a hydrogen acceptor to provide for the dehydrogenation of two molecules of glycerol. Kluyver and Braak (personal communication) suggest the formation of glyceric aldehyde as the first change. The glyceric aldehyde would then undergo fermentation.

Escherichia coli does not ferment glycerol in a mineral medium under anaerobic conditions whereas the addition of a suitable hydrogen acceptor such as peptone, asparagine, acetaldehyde, or fumaric acid starts the fermentation.

G13. The Influence of Cations on Bacterial Sporogenesis in a Liquid Medium. F. W. FABIAN AND C. S. BRYAN, Department of Bacteriology, Michigan State College, East Lansing.

The four bacteria studied were: *Bacillus subtilis*, *Bacillus cereus*, *Bacillus mesentericus* and *Bacillus megatherium*. A study of various media showed that 1 per cent peptone did not stimulate sporogenesis within 18 hours. To this basic medium were added the chlorides of the following monovalent cations Na, K, NH_4, Li (also Na lactate); Mg, Mn, Ba, Co, Pb and Ni of the divalent cations; Al, Ce and Fe of the trivalent cations and Sn of the tetravalent cations. Stock solutions of definite molality of the salts were prepared and tested for sterility. The desired molality was then prepared by adding this stock solution to a sterile stock solution of 2 per cent peptone so that the final concentration of the peptone was 1 per cent. Preliminary tests were made with all the salts to determine the viability of the organisms at the various molalities. Three molalities were then chosen for the tests; one at the point of maximum stimulation; one on the toxic side; and a third at a point less than maximum stimulation. The influence of pH on sporogenesis was determined at pH values of 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5.

The number of spores was determined quantitatively by Breed's method using Anjeszky's method for staining. The results obtained would indicate that:

1. Cations of monovalent chloride salts exert a distinct stimulating influence on sporogenesis in aerobic sporogenic bacteria in a liquid medium.
2. Cations of divalent, trivalent and tetravalent chlorides do not stimulate sporogenesis in aerobic sporogenic bacteria in a liquid medium.
3. In the presence of monovalent cations, spore formation is most abundant at the point of maximum growth. This would indicate that sporogenesis is not due to a deficiency of nutrient materials or to an accumulation of metabolic products as has been postulated in the past.
4. The pH of the medium studied did not materially affect the formation of spores within a favorable growth range, pH 5.0 to 7.5. However, an acid reaction was slightly more favorable for their formation.
5. The influence of cations at the different concentrations on the viability of the 4 bacteria studied corresponds to a remarkable degree with that for *Escherichia coli* as previously determined by Winslow and his students. This work, therefore, confirms and extends our knowledge of the physiological effect of cations upon bacterial viability.

G14. The Influence of High Frequency Alternating Current on Bacteria.

F. W. Fabian and H. T. Graham, Departments of Bacteriology and Physics, Michigan State College, East Lansing.

The apparatus used in this study was the "push-pull" self-excited oscillator and the resonating circuit for the utilization of its energy in connection with a combination cooler and condenser. The biologic influence of wave-lengths of 20, 30 and 40 meters on animals and bacteria was studied. Only the results secured with bacteria are presented here. For the experimental work with bacteria a 24-hour broth culture of *Escherichia coli* was used. Transfers were made in broth at 24-hour intervals for at least 2 days before the experiment. The number of bacteria at the beginning of the experiments averaged 150 million. This number was obtained by diluting a 24-hour broth culture about 3 times with broth. The pH value was adjusted to 7.0 each time with NaH_2PO_4.

The first series of experiments was carried out by placing the culture in a petri dish which contained a glass coil through which water circulated to maintain a temperature of 19°C. The culture was then exposed to a displacement current of 0.08 amperes at a frequency of 10 meg. cycles per second for a period of 3 hours. The technic for the second series of experiments was somewhat different; 20 cc. of culture prepared in the same way was placed in a cooler condenser and subjected to a displacement current of 0.8 amperes at frequencies of 7.5, 10 and 15 meg. cycles per second respectively. All experiments were run with suitable controls and in this series both with and without controlling the temperature. The effect on bacteria of a high frequency current at the different frequencies and intensities was determined by triplicate plating of the culture on standard nutrient agar just before the oscillator started and at 2-hour intervals thereafter for a period of 8 hours. All conclusions given are based upon data from at least 5 and in some cases 8 experiments.

Summary. 1. Bacteria as represented by *E. coli* were more resistant to the lethal effect of high frequency displacement current at the same intensity than were any of the animals studied. 2. In a high frequency displacement current of 10 meg. cycles and an intensity of 0.08 amperes the number of *E. coli* increased nearly 300 per cent during a period of 3 hours. When the intensity of the displacement current was increased approximately 10 times, the lethal effect of the current became evident. 3. Of the three frequencies studied, 7.5, 10 and 15 meg. cycles, 10 meg. cycles was the most effective, 7.5 meg. cycles was the least effective in killing bacteria while 15 meg. cycles occupied an intermediate position. 4. The high frequency displacement currents produced a regular order of death in bacteria. When the logarithm of the number of bacteria surviving at regular intervals was plotted against time, a typical survivor curve resulted.

G15. The Effect of Various Substances upon Fat Production by B. megatherium, B. mycoides and B. albolactis. J. CARVEL SPARROW, Dartmouth College, Hanover, N. H.

Three common bacteria, *B. megatherium*, *B. mycoides* and *B. albolactis*, which show distinct fat globules when cultivated on ordinary media were grown in 15 variations of Vegex agar (3 grams Vegex, 5 grams peptone, 15 grams agar, 1000 cc. water) to determine the effects of variations in the media on fat production. These variations were secured by making the following additions to 100 cc. of ordinary Vegex agar, except in No. 12 as stated in the parenthesis. *1*, vitamin A and D, 1 tablet of Adex; *2*, vitamin B, 1 gram Vegex extra; *3*, vitamin C, 2 cc. orange juice; *4*, 1 gram NaCl; *5*, 2 grams NaCl; *6*, 1 gram $CaCl_2$; *7*, 2 grams $CaCl_2$; *8*, 1 gram peptone and 1 gram sucrose; *9*, 1 gram peptone; *10*, 1 gram peptone and 5 grams sucrose; *11*, 1 gram glycerol; *12*, 3 grams Vegex and 5 grams peptone (to 1000 cc.); *13*, 0.01 gram $FeCl_3$; *14*, 1 gram glucose; and *15*, 1 gram lactose.

Qualitative fat determinations were made microscopically, the methods of Meyer and of Eisenberg being found more satisfactory as the stained fat globules were more distinct and the results more comparable with the quantitative results. For the quantitative determinations the organisms were grown in bottles containing 25 cc. of medium and having an available growth surface of 48 cm.^{2} Each bottle was seeded with 0.5 cc. of a vigorous 24-hour culture and incubated for 1 week at 37°C. The organisms were washed from the surface of the agar with distilled water. The washings were filtered and the cells were repeatedly washed with distilled water to remove as much of the medium as possible. The bacterial mass collected in the last filtration was dried on a water bath, weighed and then extracted with ether by the method of Meigs; 5 cc. distilled water and 10 cc. of 95 per cent alcohol were added and again shaken for 5 minutes. The ether layer containing the fat was carefully removed and evaporated to dryness. It was then placed in a desiccator for several hours and weighed to constant weight.

Comparison of the results obtained with these 16 kinds of media shows that there is no parallelism between growth (total dry weight) and fat production (either total fat or fat percentages). This may mean that some substances stimulate cell metabolism but not fat synthesis and storage and we might so interpret the results with lactose which trebled the weight of *B. megatherium* 50 per cent but decreased the fat content 30 per cent and the fat percentage 50 per cent. On the other hand gains in fat content and fat percentages occur in cultures

where the total weight falls far below that of the controls, increases of 100 to 300 per cent in fat percentage occurring in cultures weighing but $\frac{1}{6}$ to $\frac{1}{2}$ of the control weight. Such results were obtained for *B. megatherium* with peptone, $FeCl_3$, glucose, glycerol, and extra Vegex and peptone; *for B. mycoides* with vitamin C and peptone; and for *B. albolactis* with vitamin C, $FeCl_3$ and glucose. Such results suggest that cell fat may represent katabolic processes rather than synthesis and storage. Marked increase in fat percentage with growth or total weight equalling or exceeding the controls was found only for *B. megatherium* and *B. mycoides* in the medium to which 1 per cent peptone and 5 per cent sucrose had been added.

G16. Gelatinolysis by Aerogenic Anaerobes. G. L. Stahly and C. H. Werkman, Iowa Agricultural Experiment Station, Ames.

The ability of species of aerogenic anaerobes to liquefy gelatin has not been clearly determined. The gelatinolytic properties of 22 species and strains have been determined by 3 different procedures. In each case the method has been refined or improved in such a manner as to give clear-cut results.

1. Use of a simple culture-tube viscometer designed for anaerobic aerogenic bacteria and permitting continuous viscosity determinations during development of the culture in a gelatin medium (2.0 per cent gelatin, 0.4 per cent glucose, 0.1 per cent K_2HPO_4, 1.0 per cent peptone). The culture-tube viscometer consists of a bulb sealed to a 1 mm. capillary tube which is inserted to a fixed depth into the medium. Gas escapes through another tube provided for the purpose. An ordinary culture-tube serves as a container for the medium. Calibration marks are scratched above and below the bulb where the capillary is constricted. The method is convenient and quantitative.

2. Mercury-protein precipitation method, an adaptation of Frazier's technic to anaerobic aerogenic bacteria. Slants (0.4 per cent gelatin, 0.4 per cent glucose, 0.5 per cent peptone, 0.1 per cent K_2HPO_4, and 2.0 per cent agar) are prepared, 5 cc. active inoculum are added, with incubation for 48 hours. The slant is removed and washed in water and placed in a bath of 15 grams $HgCl_2$, 20 cc. concentrated HCl and 100 cc. water. Liquefaction is indicated by a clearing of the medium, absence of liquefaction by the development of an opaque medium.

3. Maximum temperature of gelation. Medium is composed of gelatin 10 per cent, peptone 0.5 per cent, glucose 0.4 per cent, K_2HPO_4 0.1 per cent, incubated at 37°C. Liquefaction is determined at inter-

vals by immersing tubes in a water bath in which the water is being cooled at the rate of 1° every 10 minutes. Cultures were periodically inclined to the horizontal position and the gelation temperature noted as that point at which the medium fails to break from wall of tube.

The three methods showed complete agreement. In routine determinations it is recommended that the mercury-protein and viscosity procedures be used. Graphs showing viscosity-time curves and photographs illustrating the mercury-protein method were shown and discussed.

Viscosity determinations are expressed as the ratio:

$$\frac{\text{time of outflow after inoculation}}{\text{time of outflow before inoculation}}$$

Rejuvenation of gelatin was unnecessary when the medium was incubated at 37°C. for 48 hours. If the organism is not a liquefier the ratio remains 1 or very near to 1 unless it is a slime-producing form when the ratio becomes greater than 1. Non-liquefiers did not yield a ratio less than 0.98 (*Cl. pasteurianum*) and liquefiers gave no value higher than 0.863 (*Cl. acetonigenum*). The differentiation is clear-cut.

G17. A Semi-solid Medium for Anaerobic Cultivation. ROBB SPALDING SPRAY, West Virginia University Medical School, Morgantown.

Semi-solid media for anaerobes are described by Lignières, Murray and others. Their ease of preparation and general utility, however, seem not to be as widely appreciated as deserved.

Prepared according to the following method incredibly luxuriant growths of the common anaerobic bacilli are obtained in 24 hours, without the inconvenience of any form of hydrocarbon seal,—a great advantage in staining and inoculating.

Boil 50 grams Difco Dehydrated Liver in 1 l. tap water and filter infusion through cotton by suction; make to 1 l. and adjust to pH 7.4. Add to this 37 grams Difco Brain Heart Infusion powder, 50 cc. Wolf's casein digest, 2.5 grams shred agar. Boil to dissolve agar; make to 1 l.; check titration; filter through cotton, tube deep (12 cc.) and autoclave as usual.

This medium is semi-fluid when cold, and may be transferred easily by capillary pipet. Stock cultures of anaerobes retain viability at least 30 days, making it suitable for subcultures from permanent cultures in deep brain or meat.

Stains from this medium show none of the confusing artefacts so common in smears from old brain and meat cultures. Rendered sugar-free with colon bacillus it serves as an excellent base for fermentation tests. With suitable indicator (Phenol Red) the reactions are easily read, and gas is determined by bubbles in the soft coagulum.

G18. Agar Coagulating Bacteria. A. P. HITCHENS, Fort Sheridan, Illinois.

When agar is mixed in low percentage with fluid culture media, at about 100°C. and then cooled, it reaches the limit of its swell. At a concentration of about 0.1 per cent the thin gel occupies about nine-tenths of the volume of fluid. Certain bacteria growing in such culture media cause the agar to contract and form a small opaque or semi-opaque mass resembling a coagulum. Some of the conditions which produce this contracted reaction or coagulation of agar have been observed.

G19. The Effect of Physical Environment on the Cultural Character of Bacillus mycoides. E. G. HASTINGS AND H. E. SAGEN, University of Wisconsin, Madison.

The following aspects of physical environment have been studied as to their effect on the macroscopic appearance of the growth on the surface of an agar medium: (1) Firmness of substratum; (2) character of the surface of the substratum; (3) strains in the medium; (4) orientation of cultures in motion and quiescent with respect to different axes.

On agar of usual firmness, the growth spreads from the point of seeding in the form of coarse threads, which curve counter-clockwise, producing a symmetrical pattern. On less firm agar, the pattern disappears, and the growth is diffuse.

On a surface produced by cutting a block of agar, or on the surface formed in contact with the glass, the growth is diffuse rather than patterned.

Strains induced by twisting a half cylinder of agar throw the filaments into parallel lines. Other physical factors such as motion in certain planes tend to distort the normal symmetrical pattern of growth.

G20. Comparison of a Modified Eijkman Test with Standard Methods in the Detection of Fecal Pollution of Drinking Water. W. L. WILLIAMS, R. H. WEAVER AND M. SCHERAGO, Department of Bacteriology, University of Kentucky, Lexington.

A modified Eijkman medium has been devised which has the following composition: peptone (Witte) 0.5 per cent, beef extract 0.3 per cent, dextrose 0.5 per cent. This medium is used for 1 cc. portions of water; a double strength medium is used for 10 cc. portions; and it has been shown to be superior to the original Eijkman medium in that all strains of fecal *B. coli* grow and produce gas in it at 46°C. Confirmatory tests also are more reliable when made from this medium, due to the low acid production of *B. coli* strains growing in it.

An examination of 751 water samples by the modified Eijkman and by the Standard Methods tests has shown the former to have certain advantages over the latter, viz.: (1) the presumptive test is shortened to 24 hours; (2) many samples giving false positive presumptives by the Standard Methods are negative by the modified Eijkman test; (3) many positives due to *B. aerogenes* are eliminated by the modified test. (4) This test is more reliable for the detection of fecal *B. coli*. When compared with the Standard Methods test, it identified all of the samples shown by the Standard Methods to contain fecal *B. coli*. In addition, fecal *B. coli* was confirmed from 12 samples that were negative by the Standard Methods test.

G21. The Advantages of O'Meara's Modification of the Voges-Proskauer Test. RALPH P. TITTSLER, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, New York.

A comparative study has been made of O'Meara's method for performing the Voges-Proskauer test and that approved by the American Public Health Association, "Standard Methods of Water Analysis," 6th edition, 1925. O'Meara's modification, presented in 1931, employs the addition of approximately 25 mgm. of creatine and 5 cc. of a 40 per cent potassium hydroxide solution to each 5 cc. of culture, followed by repeated shaking of the mixture. A positive test with this technique is indicated by the appearance of an intense pink color within a few minutes.

In this comparison 25 strains of *Aerobacter aerogenes*, 20 strains of *Aerobacter oxytocum* and 35 strains of *Aerobacter cloacae* were subjected to the two procedures. With each of these 80 strains a positive Voges-Proskauer reaction could be elicited. Incubation was carried on for various periods of time in Clark and Lubs' glucose-phosphate-peptone medium.

The results obtained with O'Meara's modification always correlated

with the methyl red test, the utilization of citrate and uric acid, and the fermentation of cellobiose more closely than did those of the "Standard" test. Furthermore, in every instance, this modification gave not only a more intense color, which greatly facilitated reading, but also yielded this reaction more rapidly, thereby permitting an answer within 20 minutes. Variations in the incubation period influenced O'Meara's method less than the "Standard;" that is, fewer strains became negative as the period of incubation was prolonged, and a greater number of strains yielded a positive reaction after a short period of incubation. The effect of time may be seen in the following summarized table showing the percentage of strains giving a positive reaction.

	Period of incubation in days							
	0.5	1	2	3	4	5	6	7
O'Meara's	99	99	98	85	80	71	66	61
"Standard"	88	94	86	63	56	55	55	55

G22. The Present Status of Bacterial Nomenclature. R. E. BUCHANAN, Iowa State College, Ames.

A consideration of the status of international agreements, of the policies of the International Committee, and of recent advances in systematic bacteriology. It is concluded as a result of the analysis that even the more radical views of pleomorphism, complex life histories, etc., will have only minor effect upon nomenclature and should not materially retard our stabilization of names.

G23. Suggestions for the Classification of the Genera Micrococcus, Sarcina, Neisseria, and Streptococcus. ERNEST PRIBRAM, Loyola University School of Medicine, Chicago.

The present position of the spherically shaped microörganisms is rather uncertain. There is, however, no doubt, that their differences are caused by a process of adaptation which is analogous to that of the rod-shaped representatives of the class Schizomycetes.

The genera Micrococcus and Sarcina are the lowest (most undifferentiated) representatives of the corresponding Algobacteria. The characteristics of Algobacteria: Cells and colonies are adapted to life in water. Single cells are often freely motile, by polar flagella or oscillation, rarely by peritrichic flagella (Transition forms). Cell unions form dense colonies, new colonies are often formed by swarming single cells. There is a tendency to form water-insoluble products, either in the cell (deposits of pigment, of iron, sulphur, calcium, etc.), or of water-insolu-

ble condensation products, such as gelatinous capsules, membranes, etc., surrounding and protecting the colonies. The metabolism is adapted to water-soluble and diffusible substances. Higher compounds cannot be utilized.

Order: Micrococcales (spherical and ovoid cells, usually Gram-positive).
Family: Micrococcaceae (division in all directions).
Genera: Micrococcus, Rhodococcus, Rhodocapsa, Thiocapsa, Thiosphaera, Thiosphaerion, Thiocystis, Lamprocystis, Sarcina, Thiosarcina.
Family: Pediococcaceae (division in two, or one direction).
Genera: Pediococcus (tetragenus), Lampropedia, Thiothece, Thiopolycoccus, Thioderma, Amoebomonas (cocciform type of Amoebobacter), Rhodothece, Rhodonostoc, Thiophysa.
(Arrangement according to the tendency to form pigment, to deposit condensation products, to form capsules, gelatinous masses, of swarming, etc.)

The genus Neisseria is highly adapted to human protein and may be classified with the Gram-negative Pasteurellaceae: Pasteurella, Brucella, Hemophilus. The genus Streptococcus has a typical transition form, *Streptococcus mesenterioides*, which still has characteristics in common with the Algobacteria, and may be considered a transition between Rhodonostoc and Streptococcus ("Leuconostoc"). The other streptococci, however, are much more adapted to the animal proteins and carbohydrates. They have so many characteristics in common with the Lactobacillus (Plocamobacterium), that they have been classified by Orla-Jensen with this genus, a classification which seems to be correct.

(Further details of this Classification will be published in a monograph: Klassifikation der Schizomyceten mit besonderer Berücksichtigung der Bakterien der Wiener Mikrobiologischen Sammlung, Deuticke, Wien, 1933.)

G24. A Systematic Study of the Mucosus-Capsulatus Group, with Special Reference to the Biological Position of Klebsiella pneumoniae and Aerobacter aerogenes. CARROLL W. GRANT AND LEO F. RETTGER, Department of Bacteriology, Yale University, New Haven, Conn.

In a study of the generally recognized types of the Mucosus-capsulatus group an attempt was made to identify types of mucoid organisms isolated from human throats. The type species considered were *Klebsiella pneumoniae, Kl. rhinoscleromatis, Kl. ozenae, Bacterium mucosus-capsulatus*, and *Aerobacter aerogenes* of soil and fecal origin.

The carbon and nitrogen metabolism of mucoid forms was given special attention, and an effort was made to correlate the results of such studies with the inhibitory effect exerted by certain dyes and with the serological groupings as evidenced by cross-agglutination reactions.

Differences were discerned whereby the respiratory types may be differentiated from the soil and intestinal types. The mucoid organisms isolated from human throats did not constitute a single group, but were, as may be expected from a source which is so accessible to outside contamination, of a heterogeneous nature.

No valid evidence was obtained whereby the genus *Klebsiella* may be distinguished from the genus *Aerobacter*, and it is suggested that, but for the question of priority, the generic term *Encapsulatus* given by Castellani and Chalmers be regarded as more fitting than *Klebsiella*.

G25. A Systematic Study of the Fusiform Bacteria. LAWRENCE W. SLANETZ AND LEO F. RETTGER, Department of Bacteriology, Yale University, New Haven, Conn.

Very few systematic studies have been made of the group of non-sporulating anaerobes known as fusiform bacteria. Our knowledge of these organisms has been surprisingly limited and it may be said that to many bacteriologists they are still almost entirely unknown. This general lack of knowledge is due, in a large measure, to the extreme difficulty which has been encountered in their isolation and cultivation.

During this investigation, methods and media for the isolation and cultivation of fusiform bacteria were given special attention. For securing anaerobiosis, the method of exhausting anaerobic jars by means of a vacuum pump, and of subsequent rinsing of the jar with carbon dioxide, was found to be especially suited for the cultivation of these organisms.

A selective medium for the isolation of pure cultures was developed which proved to be highly efficient. All of the strains under study were found to exhibit a marked resistance to gentian violet; this dye proved to be of particular value, therefore, in the primary isolation work. Detailed studies were carried out to determine the most suitable media for cultivation. Aqueous extracts of vegetables were found to stimulate growth. Potato extract was particularly suitable for general culture work, and a standard method was developed for the preparation of potato extract media. Potato gelatin medium served admirably for the preservation of stock cultures.

A study of the influence of gaseous environment on the growth of fusiform bacteria indicated that added carbon dioxide definitely stimulates the growth of certain types. Two per cent carbon dioxide was always introduced into the anaerobic jars, in cultivating the organisms.

Considerable attention was given to the distribution of this group of anaerobes, and attempts were made to isolate strains from as many different sources as possible. Isolations were made successfully from the normal teeth of human subjects, monkeys, dogs and rabbits; also from cases of Vincent's angina, from lung abscess and from a tonsillar lesion. No cultures were obtained from the normal teeth of cats, guinea pigs, rats and mice that were examined nor from human and animal feces. A comparative study was made of 53 strains which were isolated from various sources. It was possible to divide them into 4 different types, based upon their morphological, cultural and biochemical characteristics. The agglutination reaction was found to be unsatisfactory for classification purposes. The term "Fusobacterium" was adopted as the generic name for designating the various types studied.

Pathogenicity of certain strains was indicated as a possibility. Some evidence was obtained also which pointed to the transformation of fusiform bacteria into spirochetal forms; but these observations are incomplete and will be continued in future studies.

G26. The Systematic Relationship of Actinobacillus. LUTHER THOMPSON, Mayo Clinic, Rochester, Minnesota.

A study has been made of three strains of *Actinobacillus lignièresi* isolated from cattle in the United States, 2 strains of *B. whitmori* received from abroad, and 3 strains of *Pfeifferella mallei* obtained from laboratories in this country. The 3 organisms are responsible for disease of animals which may be transmitted to man. *Actinobacillus lignièresi* is the cause of a large percentage of the so-called actinomycosis of cattle in this and other countries. *B. whitmori* is the cause of a disease of rodents, and also of a highly fatal disease of human beings in Southeastern Asia. The close relationship between *B. whitmori* and *Pf. mallei* has been pointed out by Stanton and Fletcher. The object of this study is to show that *Actinobacillus lignièresi* bears a generic relationship to these organisms, and to confirm the report of Stanton and Fletcher.

It has been found that the 3 organisms are similar morphologically, and that the cultural differences are not greater than often occur among

species in a single genus. The serological study shows that rabbit immune sera prepared with the 3 different organisms have group agglutinins, and give a strong complement-fixation reaction with a standard glanders antigen.

The results of this study confirm the report of Stanton and Fletcher concerning the close relationship existing between *Pf. mallei* and *B. whitmori*. It is also shown that *Actinobacillus lignièresi* is related to the above named organisms morphologically, culturally and serologically The generic name Actinobacillus Brumpt 1910, has priority for this group of organisms and is recommended. It is also recommended that the genus Actinobacillus be removed from the family Actinomycetaceae and be placed in the family Mycobacteriaceae in place of the genus Pfeifferella which it supplants.

G27. Further Studies on the Life Cycle of the Avian Tubercle Bacillus.

R. R. Mellon, Ruth D. Richardson and L. W. Fisher, The Western Pennsylvania Hospital, Institute of Pathology, Pittsburgh.

The primary object of this paper is to indicate briefly that the experimental high lights of a life cycle for the tubercle bacillus have been consummated. The cycle at present consists of 4 stabilized stages which are vegetative, and 3 transition stages between them which are reproductive or gonidial,—7 in all. Of the reproductive stages 2 are probably asexual, and inquiry into the *possible* sexual nature of the other will be reported in the following paper.

Each important stage has been thoroughly single-celled before progression to the next stage has been induced by germination of its appropriate gonidial form, which form, constitutes the cytologic mechanism for the transition. For this reason Stages 2, 4, and 6 should perhaps be designated as "phases"[1] except for possible confusion in the text. Stage 1 is the S form of the avian tubercle bacillus which gives rise to Stage 2, which is filtrable. It in turn germinates as Stage 3 in stabilized diplococcus form. A transition form of Stage 3 is the large tetrad form, Stage 4, which gives rise to the stabilized diphtheroid State 5, which in turn yields acid-fast gonidia constituting Stage 6. These latter then germinate as the R form of the tubercle bacillus (Stage 7) and then presumably to the original S form. The cycle's completion

[1] The term "phase" indicates an absence of detectable multiplication of such a form before its germination into a "stage," which is capable of vegetative multiplication for many "generations."

rests only on transformation of the R to the S form. But since the latter has been demonstrated by others for the avian bacillus, and since the R form constitutes a recognizable stage of the tubercle bacillus, the cycle is virtually complete. Stages 1, 6, and 7 are acid-fast; Stages 2, 3, 4, and 5 are non-acid-fast.

It is noteworthy that the stabilized diphtheroid Stage 5 was transformed to the R form of the tubercle bacillus (Stage 7) on 6 occasions by reason of breaking the dormancy of the acid-fast gonidia, by special methods to be detailed later. Most of the observations indicate its origin from the acid-fast, spore-like gonidia, although it is not certain that it is limited to these. It appears that these coccoidal structures throw out a germ tube, as does a fungal spore, which elongates into an acid-fast filament, which simultaneously fragments to ordinary tubercle bacilli.

We have previously shown that the non-acid-fast filtrable phase of a saprophytic acid-fast bacillus may revert to the original directly, or by way of the diphtheroid without its stabilization. But the interpolation of several stabilized non-acid-fast cyclostages between the filtrable form and the original is now shown for the tubercle bacillus. This establishes conclusively the reproductive or gonidial status of the filtrable form, which cannot be of "involution" nature.

In these two important particulars the study represents a fundamental departure from Kahn's which is exclusively morphologic. The probable significance of Kahn's pleomorphic study is that it foreshadows the existence of a true life cycle and in principle is quite in accord with the previous study of one of us (Mellon, 1925) for a diphtheroid bacillus. But the proof of a true life cycle can only come by the demonstration of gonidial or reproductive forms; which is chiefly accomplished in this instance by their germination, *not into the original form, but into variants.*[2] Moreover, these stabilized variants (in this case, distinct species, so-called) must return to the original acid-fast form.

Although conclusive evidence for a sexual cycle is yet to be obtained, examination of a chromosomal mechanism (Proc. Soc. Exper. Biol. and Med., **30**, 110) discovered for a stage in this cycle points more strongly than ever in this direction. On this working theory that the variation concerns itself with a genetic mechanism, it is possible to postulate new

[2] The morphology of such variants finds their prototypes in the pleomorphic cycle, (Kahn), but monomorphic theory refers to them simply as *pleomorphic* forms, since they are not capable of dissociation from a pure culture under "normal" conditions.

or unusual types of tubercle bacilli as the result of segregative and hereditary recombinations.

Such a strain has, in fact, been encountered in our experiments. It produces a type of granuloma in rabbits, whose tuberculous character is recognized difficultly, if at all. The giant cells, although numerous, are distinctly not of the Langhans type, but more of the Dorothy Reed type of Hodgkin's Disease. However, they contain numbers of acid-fast bacilli varying from a few to hundreds! The entire reticulo-endothelial system of the liver seems given over to their production.

Summary. Dissociation of the tubercle bacillus into a heterogeneous non-acid-fast progeny and its occasional reversion to the original is a very old observation. The S and R variability reversions are more recent, while demonstration of a filtrable phase (granules of Much and their congeners) for pure cultures is quite recent. The sum total of these observations constitutes a collection of "variability fragments," so to speak; but it requires orderly allocation by experiment to dispel the chaos that they have represented to monomorphic theory.

These studies represent, we believe, the first experimental integration of these several "variability fragments" in accordance with a single unifying conception, namely, the biologic sequences of a true life cycle. Single-celled cultures only have been employed. The variability of the non-acid-fast progeny is of such a character as to suggest strongly its implication with the genetic mechanism of the cell, which accounts in part for the following paper.

G28. Observations on a Chromosomal Mechanism for the Bacterial Cell.
CARL C. LINDEGREN AND RALPH R. MELLON, Institute of Pathology, The Western Pennsylvania Hospital, Pittsburgh,

Mellon (1918) working with diphtheroids indistinguishable from those encountered in the tubercle cycle showed that macrotetrads are definitely identified with the transformation to diplococci and *vice versa*. Mellon, Richardson and Fisher (1932) have demonstrated macrotetrads play an important part in the life cycle of the tubercle bacillus. Lindegren and Mellon (1932) have described nuclear phenomena in the development of these macrotetrads in a preliminary paper. Observations on the chromosomal mechanism have made possible the formulation of a working hypothesis to the effect that the macrotetrads result from meiosis of a zygote nucleus. It is necessary, however, to rule out the alternate possibility that the small vegetative tetrads enlarge directly to a macrotetrad. Fifteen macrotetrads from a culture 6 weeks old were

isolated and planted on nutrient agar. Ten of these produced colonies a few days later and in a week all the colonies contained macrotetrads. In one case one unit of the tetracyte was separated from the other three and single colonies were produced from each. Both contained macrotetrads, indicating that no sex factors were segregated and that a typical bi-sexual mechanism does not exist. The mass of tetrads germinate to produce small diplococci; 68 such diplococci, or strings of cells obviously originating from a single diplococcus, were isolated from a 4-day culture and 33 produced colonies. All these colonies contained macrotetrads. Actually 12 of these colonies grew from single diplococci. The difference in viability of the macrotetrad and the diplococcus is significant.

Cytological study shows that the vegetative diplococcus may divide to produce a small tetrad. This usually breaks up to form 2 diplococci but may remain together, and continued division produces a short chain. In these vegetative cells no nuclear structure is discernible. But some diplococci enlarge and a chromosome appears in each half. The evidence suggests that the intervening wall is penetrated by one chromosome and the zygote formed in the adjacent cell, leaving one empty cell; or the intervening wall appears to be penetrated by both chromosomes simultaneously and dissolves, resulting in autogamous fusion.

The enlarging chromosomes stretch out to occupy both halves of the original diplococcus. Both chromosomes synapse and begin to show a segmental structure. At first, only 2, then 3, 4, and 5 segments appear in each chromosome. These segments are closely paired. Finally, when synapsis is complete a string of 7 chromomeres appears. These chromomeres begin to stain less intensely and a resting nucleus containing as many as 20 net knots is found. Following this stage chromatin again appears in the cell but now about 14 chromomeres are visible in 2 separate denser strands with many finer interconnecting threads. The 2 main strands contract and a decreasing number of segments are discernible until finally only 2 small unsegmented chromosomes appear within a small nucleus.

One chromosome always appears a little smaller than the other, but whether this is true heteromorphism or merely a difference in the rate of contraction is difficult to ascertain. The stained slides suggest these 2 chromosomes pass to opposite halves of the cell, and it appears in some figures that centrosomes and centrioles are present in the cell at this time. No spindle fibers were detected to date. A cell wall forms between the 2 chromosomes. Each forms 7 chromomeres and eventu-

ally 2 shortened chromosomes appear in each half. These pass to opposite sides of the cell and another cell wall is formed producing the macrotetrad. Demonstration of the nuclear structures was made possible by the use of acetocarmine, brazilin, and hematoxylin techniques of the modern cytologists specific for the chromosomal mechanism.

G29. Some Effects of Salt on the Morphology of Bacillus megatherium. (With motion pictures.) OLGA A. SMITH, Lawrence College, Appleton, Wisconsin.

Five variants of a stock culture of *Bacillus megatherium* which has been cultivated for more than a dozen years on Vegex broth and Vegex agar (Broth: Vegex, 3 grams; peptone, 5 grams; water, 1000 cc. Agar: 15 grams of agar per liter of broth) were repeatedly transferred to Vegex broth and agar varying in salt content as follows: no added salt, 1, 2, 4, 6 and 8 per cent added salt. The differences in pH (which were not corrected) varied with the salt content; the initial broth without added salt having a pH of 6.6, the 1 per cent salt a pH of 6.8, and serially, 7.0, 7.4, 7.8 to 8.0 for the 8 per cent salt broth. Observations were made after 1, 2, 4 and 6 days at 33°C.

Among the results observed with the 5 strains studied are the following: (1) The induced changes were not permanent, but were evident responses to the current conditions. (2) The changes here reported were more marked on agar media than in broth. (3) As the salt content increased, the colony color changed from cream to darker cream, to red brown and finally to yellow. (4) The 1 per cent salt media gave rough colonies and long slender cells. Smooth colonies and shorter cells were characteristic of media without added salt and with all salt percentages above 1 per cent. As the salt increased above 1 per cent there was an increase in colony smoothness and a marked decrease in cell length, the cells becoming distinctly coccoid (diplococci and tetrads) in 8 per cent salt. (5) The coccoid forms arise in two ways: by cross-division of the ordinary long cells and by the formation of several coccoid bodies within the long cells. (6) Spore formation was most rapid with the 2 per cent salt; above that percentage spore formation slowed as the salt increased.

G30. Independent Variation of Bacterial Properties. W. J. NUNGESTER, Department of Bacteriology and Patten Research Laboratories, Northwestern University Medical School, Chicago.

The tendency in dissociation studies has been to emphasize the corre-

lation in variation of bacterial properties. Although such correlations are without doubt often true, many instances of independent variation of bacterial properties have been reported in the past. The independent variation of bacterial properties must be considered in interpreting the whole phenomenon of bacterial dissociation. It appears to the author that this type of variation does not support the concepts involved in the hypothesis of life cycles but forces one to look elsewhere for an interpretation of the general phenomenon of bacterial variation.

Because of this view concerning the importance of independent changes in bacterial properties the author presents his experiences on this subject. These include investigations covering such subjects as: (1) alterations in virulence of *B. anthracis* variants without apparent changes in colony form, (2) independent variation of colony form and acid production from lactose among variants of a coli-like organi , (3) alteration in acid production from lactose without variation in colony form in organisms giving a delayed acid production from this sugar and (4) a lack of correlation in colony structure and gas formation from glucose broth among variants of *B. paratyphosus B.* (5) The final study of this type deals with an investigation of a variant (C-16) obtained from a recently isolated coli-like organism. This culture gave a delayed acid production from lactose, maltose and sucrose. It could be trained to produce acid readily from each of these sugars by serial cultivation in liquid mediums containing the particular carbohydrate. By using this specific method of stimulating change, a group of variants has been trained with all possible combinations of rapid acid production from the 3 carbohydrates used. The 7 variants thus obtained agglutinated in a dilution of 1:2560 with an anti-serum prepared against the parent form. All gave rise to colonies essentially of the R or RS type.

G31. A Variable Proteus-like Organism Fermenting Carbohydrates without Gas Formation. H. J. Sears and Martha Rohner, Department of Bacteriology, University of Oregon Medical School, Portland.

A Gram-negative bacillus showing the variable morphology and the swarming characteristic typical of *Proteus vulgaris* was isolated from tissues at autopsy. It was probably a post mortem invader. In mice, rabbits and guinea pigs it showed no significant pathogenicity. It ferments glucose, galactose, levulose, mannose, mannitol and salicin in 24 hours. Acid is formed but no gas. Acid is produced late in liquid media containing sucrose, inositol and raffinose; in the first two this

occurs in 3 to 4 days, in the last only after 3 weeks' incubation. On agar media containing sucrose numerous secondary colonies are formed in 3 to 5 days. When transfers from these are made to sucrose broth abundant acid appears in the latter in 18 to 24 hours. A very small number of secondary colonies are formed on raffinose agar after several weeks' incubation. These prove to be rapid raffinose fermenters. No secondary colonies have been observed on inositol agar. Xylose, lactose, maltose, rhamnose, arabinose, dulcitol and sorbitol are not fermented. Gelatine is not liquified, indole is not formed and urea is not decomposed as by proteus strains. The organism possesses numerous peritrichous flagella.

The sucrose and raffinose variants are stable and independent of each other. They differ from the parent strain only in their action upon sucrose and raffinose respectively. They show the same morphological characteristics and cross-agglutinate with each other and with the parent strain.

By growth upon phenol agar a non-motile O variant may be produced. It reverts readily to the motile type, however, when recultured on ordinary media.

G32. Dissociation of an Acid-fast Organism. JANET MCCARTER AND E. G. HASTINGS, University of Wisconsin, Madison.

A study has been made of rough and smooth single-cell strains of an acid-fast organism. This organism was isolated in our laboratory from a mesenteric lymph node of a tuberculin-reacting cow which showed a skin lesion only upon autopsy. It is non-pathogenic for chickens, guinea pigs, and rabbits.

It has been found that the S form changes readily to the R form, and vice versa, in liquid media according to the oxygen conditions therein. Regardless of whether the inoculum has been the R or S form, if growth occurs throughout the medium, it will be the smooth type as detected by growth on Dorset's coagulated egg medium; whereas, if the growth occurs as a pellicle on the liquid medium, it will give a rough type of growth on the egg medium.

G33. An Example of Anaerobic Dissociation. ROBB SPALDING SPRAY, West Virginia University Medical School, Morgantown.

Microbic dissociation is observed in many aerobes, but has received comparatively little attention in anaerobic studies. The instance here

reported is in a strain of *Cl. putrificum* isolated in 1928 from a mixed culture of *Cl. botulinum*. Plating yielded 4 colony forms. Two proved to be *Cl. botulinum* and *Cl. tertium*, while the other 2 yielded cultures with reactions of *Cl. putrificum*. The latter have been repeatedly subcultured and plated over 2 years time, and are beyond question phases of the same organism.

The first, called "Smooth," is the usual loosely, wooly colony with dense opaque center. Stains show normal terminal sporing bacilli. Broth cultures under Hall marble seal remain uniformly turbid.

The second, called "Rough," shows only as a perceptible haze in deep agar. Stains show almost solely long wavy filaments in clumps. Broth cultures quickly settle leaving a clear supernatant fluid.

The "S" type, on repeated plating and subculture, continues to give off the "R" type, while the latter retains its character with great tenacity. Repeated platings, however, occasionally show "S" colonies from which the "S" culture has been recovered.

In view of these observations during over 2 years continuous cultivation we believe it a condition analogous to the S-R cycle of the aerobes and that it should be so recorded.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

A1. What Direction Soil Bacteriology? H. J. Conn, Agricultural Experiment Station, Geneva, N. Y.

This paper presents general discussion of the lack of practical results from the study of soil micro-organisms. It is pointed out that the knowledge acquired during the last 50 years of soil bacteriology has taught us the reason why certain long established practices in soil management are necessary, but (except for the inoculation of legumes) has not introduced any new methods. The question is raised as to whether soil bacteriology is proceeding in a direction that is likely to bring practical results in the future.

This lack of practical results came to be appreciated quite strongly about 20 years ago and at that time soil bacteriologists began searching for new procedures, with result of changing completely the method of attack used in studying this field. The new methods have brought out many interesting facts concerning the behavior of bacteria in the soil, but it is still too early to decide whether or not they are going to lead to more practical findings than the earlier methods. Some of the lines of work that have been developed during the last few years are at least

promising and deserve to be kept in view by those who wish to get an idea in what direction soil bacteriology is heading.

A2. Respiration Studies on Rhizobia from Various Leguminous Plants. C. E. GEORGI, P. W. WILSON AND W. H. PETERSON, University of Wisconsin, Madison.

The respiration of members of the rhizobia species was measured under different pO_2 in a respirometer of the type described by Novy and Soule. The method used by these workers was modified in the following details: (1) The cultures were grown on a yeast-water glucose agar medium placed in one arm of an Eldredge tube. (2) The CO_2 formed was absorbed in N NaOH placed in the other arm of the tube. The initial oxygen pressures varied from less than 35 mm. to about 740 mm. After respiration had ceased, residual O_2, total CO_2 evolved, and residual glucose were determined.

The respiratory quotient of the cultures varied from 0.90 to 1.10 with the majority slightly greater than 1.00. In cultures containing 1 per cent glucose, the utilization of this source of carbon was 100 per cent; in cultures containing 2 per cent glucose, the utilization was 50 to 70 per cent. The glucose consumed which appeared as CO_2 varied with the culture and to some extent with the pO_2, but in general was 60 to 75 per cent. The rate of oxygen consumption increased moderately as the pO_2 was increased.

A3. The Effect of Nitrogen Source on Oxygen Consumption by Rhizobium. R. H. WALKER AND D. A. ANDERSON, Iowa State College, Ames.

In connection with the investigations on the nitrogen metabolism by Rhizobium, the effects of various nitrogen sources on oxygen consumption have been studied. Ammonium chloride, $NaNO_3$, urea, alanine, and yeast-extract were used as sources of nitrogen. Each of these materials was added to glucose solution in varying amounts so as to obtain media containing 0.72, 7.2, 36.0, 72.0, and 144.0 mgm. of nitrogen per liter in each case respectively. Cultures of *Rhizobium meliloti*, *Rh. trifolii*, *Rh. leguminosarum* and *Rh. phaseoli* were grown in each of the media and oxygen consumption was measured in micro-manometers by the Warburg technic.

The data secured thus far indicate that both the rate and extent of oxygen consumption by all 4 species were increased considerably by yeast-extract. The amount of oxygen consumed increased in all cases

as the concentration of yeast-extract in the medium was increased. However, the proportional increase was much larger for *Rh. leguminosarum*, *Rh. trifolii* and *Rh. phaseoli* than it was for *Rh. meliloti*.

The rate and extent of oxygen consumption by *Rh. leguminosarum*, *Rh. trifolii* and *Rh. phaseoli* were increased only very slightly, or not at all, by NH_4Cl, $NaNO_3$, urea, and alanine. On the other hand, the rate of oxygen consumption and the total amount of oxygen consumed by *Rh. meliloti* were increased by NH_4Cl, $NaNO_3$, urea and alanine over that secured in the no-nitrogen medium, but not to as large an extent as by corresponding amounts of the yeast-extract.

It is possible that the increased oxygen consumption induced by the yeast-extract may have been due, aside from the nitrogen which it contained, to one or more of the other constituents such as carbohydrates, vitamins or other organic or inorganic materials contained in the yeast-extract. The part played by these various constituents in the stimulation of oxygen consumption by Rhizobium is now being investigated.

A4. The Effect of pCO_2 on the Nitrogen Fixation Process of Certain Leguminous Plants. P. W. Wilson and E. B. Fred, University of Wisconsin, Madison.

Inoculated clover plants were grown on agar and in sand in a closed system and provided with atmospheres containing 0.03, 0.1, 0.2, 0.4, and 0.8 per cent CO_2. The effect of the pCO_2 on nitrogen fixation, dry weight, nodule formation, etc., was observed. Raising the pCO_2 from 0.03 to 0.1 per cent causes a pronounced increase in all of the foregoing characteristics of plant growth, but further increments in the pCO_2 do not result in correspondingly greater development.

In addition to an increase in the number of nodules, addition of CO_2 causes a change in their size and distribution. The nodules are larger and tend to be more evenly distributed on the root system rather than clustered on the tap root.

It has been demonstrated that the effect of added CO_2 is due to increase in the partial pressure of the CO_2 in the atmosphere and not to total CO_2 made available to the plant.

The major findings obtained with clover plants in a closed system have been verified for clover grown in bottles open to the air and for alfalfa in the closed system.

A5. Changes in the Viscosity of Solution Cultures Induced by Various Species of Rhizobium. D. A. Anderson and R. H. Walker, Iowa Stage College, Ames.

In the course of a study of the gum or polyuronide synthesized by the legume root nodule bacteria, a rapid method was sought for measuring the gum content of solution cultures of the organism. Since the gum produced by Rhizobium is an hydrophilic colloid, it was thought that it might increase the viscosity of the solution cultures to some extent. Viscosity measurements were, therefore, made on solution cultures of Rhizobium in a glucose-yeast-extract medium.

Certain of the cultures produced a high degree of viscosity which was found to be proportional to the amount of gum they contained. Other cultures, however, showed viscosities only slightly greater than that of the sterile culture medium in spite of the fact that in many cases they contained as much gum as the high viscosity cultures.

In order to determine whether or not the ability to increase the viscosity of the solution cultures is characteristic of certain species, the viscosity-producing power of 59 strains representing 8 species of Rhizobium, was determined. The results indicate that the species can be divided into 2 groups on the basis of their power to increase the viscosity of the culture medium, at least when grown in a glucose-yeast-extract solution.

The first group includes *Rhizobium meliloti*, *Rh. japonicum*, *Rh. lupini* and the cowpea bacteria. These organisms produce little or no increase in the viscosity of the medium. Taking the relative viscosity of the sterile medium as 1.00 the viscosities of the cultures of this group fall below 1.10.

The second group includes *Rhizobium trifolii*, *Rh. leguminosarum*, *Rh. phaseoli* and probably the Dalea bacteria. These organisms produce a relatively high viscosity. The ratios of the viscosities of the cultures to that of the sterile medium range from slightly more than 1.10 to nearly 2.50. Most of the values fall between 1.20 and 1.80.

These results, coupled with the findings of other investigators, indicate that the differences in the viscosity producing power of different species of Rhizobium are probably due to differences in the chemical nature of the gum rather than to mere differences in the amount of gum produced.

A6. Nodule Formation on Excised Roots of the Bean. KEITH H. LEWIS AND ELIZABETH MCCOY, University of Wisconsin, Madison.

Tissue culture of plant parts as a medium for experimentation has not been much exploited, except by botanists studying growth of the excised embryo and regeneration of plant parts. Robbins' work on the cultivation of excised roots, or root systems from which the tops have

been removed as soon as the roots have completely emerged from the seed coat, suggested an attempt to culture leguminous roots, with inoculation to induce nodule formation. Excised roots of the pencil pod black wax bean, a variety of *Phaseolus vulgaris L.*, have therefore been raised in artificial culture by an adaptation of the Wilson technique for etiolated plants, i.e., aseptically in an agar medium containing the necessary mineral nutrients except nitrogen, and with 0.5 per cent of sucrose in place of the sugar of photosynthesis. Nodulation of such excised roots, grown either alone or in combination with etiolated whole plants or tops with adventitious roots, has been accomplished. The nodules formed were histologically normal but lacked the usual starch reserve of the bean nodule.

A7. Veal Infusion as a Medium for Purity Tests of Cultures of Rhizobia.

A. W. Hofer, Agricultural Experiment Station, Geneva, N. Y.

Since the report last year on the inability of cultures of Rhizobia, except the alfalfa group, to grow in veal infusion broth, tests have been conducted to determine the value of this medium for purity tests on root nodule cultures. The work was carried out in 2 steps, of which the first consisted of the picking of colonies from plates made from commercial legume inoculants. The cultures obtained in this way were inoculated into veal infusion broth, litmus milk and potato, to determine the extent of correlation between tests on these 3 media. Most of the cultures producing atypical reactions in the various purity media were picked from plates made from humus or peat cultures. These organisms were fluorescent, were not capable of forming nodules, grew in colonies that appeared exactly like those of the root nodule organism and were, undoubtedly, counted as such in the past. Results of this study show that the plate count, as now used for determination of numbers of Rhizobia in peat and humus cultures is, at least on certain samples, incapable of giving even a close approximation to the actual count.

In the second part, colonies from culture plates prepared from soil from a clover field were picked and studied on the 3 media. On the whole, it was found that veal infusion was fully as valuable as milk and potato in determining the presence of contaminants in legume cultures. Results with veal infusion were also easier to read, as turbidity is the only characteristic that is usually considered. A further advantage lies in the fact that the veal infusion test is much more rapid than the others. When used as a test for *B. radiobacter*, this medium showed the same advantages noted above.

A8. Some Physical Factors Affecting the Determination of the Number of Nitrogen Fixing Bacteria in Soil. HAROLD W. BATCHELOR, Ohio Agricultural Experiment Station, Wooster, Ohio.

A study has been made of several physical factors affecting the apparent number of nitrogen fixing bacteria in soil as determined by the plate method. The apparent number as a function of the soil separate used as a sample has been studied. High counts are obtained from fine separates; low counts from coarse separates.

A study has been made of the effect of the total number of shakes and the rate of shaking a soil suspension on the count obtained. The total number of shakes varied from 1000 to 8000 shakes. The rates of shaking varied from 300 to 650 per minute. Within these limits a complex but relatively symmetrical relation has been found between the count obtained and the time and rate of shaking. An eightfold increase in the count has been obtained by proper shaking.

The count as a function of the time and depth of sampling the soil suspension has been studied. A relation has been found between the apparent count and the total soil surface that passes a given depth when the soil suspension is allowed to settle. The count is thus a function of the mechanical analysis of the soil, the temperature, and the time and depth of sampling the soil suspension.

It is suggested that a consideration of these and other simple physical and chemical factors may result in a marked reduction of the so-called biological error in counting soil bacteria.

A9. Calcium-precipitating Bacteria from Mud of a Fresh Water Lake. FRED T. WILLIAMS AND ELIZABETH MCCOY, University of Wisconsin, Madison.

A preliminary survey of the microflora of the bottom sediments of Lake Mendota has shown that most groups of soil organisms are represented in the deep water muds. Have these microörganisms a part in the precipitation of calcium carbonate, shown by x-ray and chemical analyses to be present in such fresh water deposits? Many aerobic and facultative bacteria have been found and several isolated, that will precipitate carbonate on artificial culture media containing either organic or inorganic calcium salts (*e.g.*, $CaSO_4$, $CaCl_2$, Ca lactate). Quantitative analyses have been made of the calcium precipitated in liquid media at different pH values and temperatures of incubation (10° and 28°C.). No fixed relation between precipitation and final pH of a culture was noted, since positive precipitation occurred between pH 7.2 and 9.4,

usually about 7.7 however. Although phosphates were present in the original artificial media, no phosphate was found in the precipitates. Carbonate precipitation by pure cultures has been demonstrated on solid medium prepared with mud extract without additional calcium.

A10. A Review of the Bacteriology of the Normal and Infected Udder. G. J. HUCKER, New York Agricultural Experiment Station, Geneva, New York.

During recent years there has been a growing tendency to attach more significance to the question of infection of bovine udders. This importance is not only from the standpoint of the public health control official and the bacteriologist, but it is also assuming an important economic significance. The relationship of the bacteriology of the udder to septic sore throat epidemics, to all phases of mastitis as well as to other pathological conditions of the udder is a problem of daily interest.

This discussion embraces a general review of the work which has been completed both upon the flora of the so-called normal udder as well as upon the organisms associated with various types of infections. The discussion primarily concerns the invasion of streptococci into the udder. The review of the literature upon this subject, to the present time is summarized and conclusions are drawn as to the most important results which have been secured regarding the bacteria in the bovine udder.

The second phase of this discussion deals with the results of recent investigations concerning the significance of the presence of long chain streptococci and of cells in milk and their relationships to pathological changes in the udder. In addition, the various types of udder infections are classified with data to substantiate the possible significance of various organisms which are found associated with each type of infection.

The incidence of the various types of mastitis is discussed, together with the relative efficiency of the various tests commonly carried out in the laboratory for the detection of milk from abnormal udders.

Possible lines of work which may be of significance in developing elimination and control methods in connection with mastitis are discussed. Certain phases of significant possibilities are outlined as possible productive results in studying udder infections.

A11. The Streptococci Found in Freshly Drawn Milk. W. D. FROST AND MILDRED A. ENGELBRECHT, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

The work presented here is subsidiary to the milk part of the work reported to this Society last year by the senior author in his paper entitled: Carriers of *Streptococcus epidemicus*. Most of the approximately 10,000 milk samples examined were group samples from about 10 cows each, although a considerable number were from individual cows. The methods of isolation and identification were those of Brown and the basis of classification that of Holman.

The quantitative studies show that streptococci are widely and persistently common in cow's milk and with a few exceptions this applies to all the different types of streptococci.

Our qualitative studies show that there are 3 quite sharply defined groups which may be defined as follows. *First group.* This is made up of the types which form typical *beta* colonies according to Smith and Brown's classical description. Included here are those important agents of human pathology that are commonly carelessly characterized as *Streptococcus hemolyticus*. We find that the following occur frequently, —*S. infrequens*, *S. mastitidis*, and *S. asalignus*, and that others, *S. epidemicus*, *S. pyogenes*, *S. equi*, *S. subacidus*, *S. hemolyticus i*, and *S. anginosus*, appear occasionally.

Second group. Beta strains vary widely in their hemolytic titre, and if the test-tube test for hemolysis is applied, as recommended in Medical War Manual No. 6, Laboratory Methods of the United States Army, p. 146, they will be split into the strongly hemolytic, or true betas, which is our first group and the weakly hemolytic forms which constitute our second group. Brown, Frost and Shaw put them in their groups 7, 8 and 9. Haynes studied them in considerable detail and came to the conclusion that they were very closely related, both culturally and serologically, to the true *beta* group, a view which we share. Cumming has recently called similar forms pseudohemolytic streptococci. The probability is that while this division into 2 groups has a practical value it has little if any taxonomic value. This group is assumed to be nonpathogenic for man. We have typed comparatively few of this group but enough to know that there are commonly present pseudohemolytic varieties of *S. mastitidis*, *S. infrequens* and *S. asalignus*.

Third group. This contains all the streptococci which are non-*beta* hemolytic and, therefore, not included in our first and second groups. The great majority of these belong to the alpha type but a few are gammas and very occasionally other blood types. Their fermentative reactions throw them into species very similar to, if not actually identical with, *S. mitis*, *S. fecalis* and *S. salivarius*. This group is more common

in milk than the *beta* types. The species named are probably the most frequent causes of bovine mastitis and apparently in the order named. These bovine forms are not known to be pathogenic for man.

A12. The Diversity of Types Present in Stock Cultures of Streptococcus epidemicus. P. R. EDWARDS AND W. W. DIMOCK, University of Kentucky, Lexington.

The cultural and biochemical characteristics of 65 stock cultures of *S. epidemicus* which had been collected from 6 laboratories were examined. It was found that 16 cultures from one laboratory and 2 cultures from a second laboratory were identical in their biochemical characters with sorbitol fermenting streptococci of animal origin. The remaining 47 cultures resembled the human type. Thirteen of the 18 strains resembling the animal streptococci were isolated from cows. The remaining 5 were said to be from human sources.

Duplicates of 4 of the 5 latter strains were obtained from the laboratories originally distributing them. All of the duplicates were of the human type. No duplicates of the fifth strain could be obtained.

It is evident that the 13 cultures isolated from cows and sent to us as *S. epidemicus* are in reality animal streptococci. The 5 cultures said to be of human origin, but which possessed the characteristics of the animal strains, evidently represent mislabelling of cultures or admixture of types. Sorbitol fermenting streptococci probably are not the cause of septic sore throat.

A13. The Growth of Pathogenic Organisms in Milk. A. M. WAHBY AND J. M. SHERMAN, Cornell University, Ithaca, N. Y.

The following milk-borne pathogenic bacteria, and related organisms, were tested: *Bacterium typhosum*, *B. paratyphi* (A), *B. paratyphi* (B), *B. enteritidis*, *B. suipestifer*, *B. dysenteriae* (Shiga), *B. dysenteriae* (Flexner), *Corynebacterium diphtheriae*, *Brucella abortus*, *Vibrio cholerae*, *Streptococcus scarlatinae*, *S. epidemicus*, and *S. mastitidis*.

As would be expected, all of these organisms grew actively in milk at 30°C., while none of them grew at 5°C. At 15°C. all grew with the exception of *Br. abortus* and *V. cholerae*. At 10°C. none grew significantly with the exception of the 4 representatives of the paratyphoid group, though the typhoid organism sometimes showed very slight growth after one week. The paratyphoids did not grow at 7.5°C.

A14. The Lysozyme Content of Milk from Various Species and Its Relation to the Fecal Flora of Their Nurslings. PAUL S. PRICKETT, NORMAN J. MILLER AND FRANCIS G. MCDONALD, Mead Johnson and Company, Evansville, Ind.

The relationship of the enzymic content of milk to the fecal flora of nurslings has received little attention. Since it has been reported recently that lysozyme, an enzyme in human milk, has a decided influence on the fecal flora of the human nursling, we investigated: (1) the milks and the corresponding nurslings' feces of various mammalian species for their lysozyme content, and (2) the fecal floras of these nurslings to determine whether there was any correlation with the lysozyme contents of the respective milks and feces.

The presence of lysozyme in feces or milk is determined by the lytic action of these substances on susceptible bacteria. The lysozyme content can be determined quantitatively either by macroscopic or by microscopic technic. Both technics were employed in this study. When sufficient material was available both were used, but only the microscopic technic was applicable when small amounts of material were available, or when the lysozyme content was too small to be detected by the macroscopic technic. Fresh feces, stained by Kopeloff's modification of the Gram stain, were used for the study of the fecal flora.

Human milk, which is rich in lysozyme, was chosen as the standard for comparison. In all, the milks of 11 species were examined. The amounts available in some cases were too small to perform any but crudely quantitative measurements. Milks from the human, monkey (rhesus), dog, cat, rat and llama all showed approximately the same lysozyme concentration. Rabbits' milk had a slightly weaker concentration. Cows' milk showed about $\frac{1}{100}$ of the lysozyme content of human milk. Milks from goats, sheep and guinea pigs contained only traces or none.

Nurslings' feces were not available from all 11 species, but of the ones examined, those from the human, monkey and dog showed the strongest lysozyme concentrations, all being about equal. Human infants fed cows' milk showed no lysozyme in their feces, whereas this enzyme was demonstrable in nursling calves' feces. Nursling rabbits' feces contained lysozyme, but those from the nursling cat, rat and guinea pig did not.

The fecal floras were studied for both their bacterial types and their Gram-positive: Gram-negative ratios. Judged by the decreasing percentage of Gram-positive organisms present, the fecal floras of the various nurslings were found to follow this order: human, monkey, dog

cat, rat, rabbit, calf and guinea pig. Based on the similarity of the bacterial types to those found in the human nurslings' feces, these various floras followed the same general order except that of the nursling rat which, next to that of the guinea pig, showed the least similarity. In general, the fecal floras of the various species' nurslings appeared to be influenced by the lysozyme content of the respective milks, and also by the lysozyme content of their feces. This influence was more discernible in the Gram-positive: Gram-negative ratios.

A15. The Relative Efficiency of Various Methods of Sterilization of Milk Utensils in the Destruction of Es. coli. A. G. LOCHHEAD AND C. K. JOHNS, Division of Bacteriology Central Experimental Farm, Ottawa, Canada.

The relative sterilizing efficiency of hypochlorite, chloramine-T, lye solution and hot water was determined through a series of tests using milking machine tubes and milk cans uniformly contaminated with sour milk having a high content of *Es. coli*. In the milking machine tests total counts of bacteria surviving revealed no great difference in effectiveness although the hypochlorite and lye showed to some advantage. On the other hand, striking differences were shown in the ability of the treatments to destroy *Es. coli*. While hot water (170°F., 20 minutes) showed the greatest destruction, lye, only slightly less effective, showed by far the greatest germicidal action against *Es. coli* of the chemical compounds used. Tests with milk cans similarly contaminated, and analyzed shortly after treatment, showed that although lye and hot water rinse were less effective against total count, the lye had by far the greatest germicidal effect on *Es. coli*. An analysis of cans 19 hours after treatment indicated that the chemical solutions were decidedly more effective than hot water in preventing bacterial development subsequent to treatment. Chlorine compounds were more effective in keeping down the total count, lye acting more specifically against *Es. coli*. In the estimation of *Es. coli* comparative studies of various media for plating and dilution methods showed the cyanide-citrate agar of Tonney and Noble and eosin-methylene blue agar to be the most generally satisfactory.

A16. Species of Escherichia-Aerobacter Organisms Responsible for Some Defects in Dairy Products. W. B. SARLES[1] AND B. W. HAMMER, Agricultural Experiment Station, Ames, Iowa.

[1] Now in the Department of Agricultural Bacteriology, University of Wisconsin.

Several defects in milk and cream, due to organisms of the Escherichia-Aerobacter group, have been studied. Pure cultures of the organisms were isolated from samples of the defective products by one or more of several methods, which included plating dilutions on standard and eosin methylene blue agars, and various enrichment procedures followed by plating.

A sample of raw cream became ropy due to *A. aerogenes*. Ropiness in samples of pasteurized milk, probably due to contamination of the milk following pasteurization, was caused by *A. aerogenes* and *A. cloacae*. *E. neapolitana* was found to be the cause of ropiness in one sample of raw market milk. An unusual case of gassiness in raw market milk was caused by *A. oxytocum*.

Gas in butter cultures and in milk set for cottage cheese was apparently due to a variety of Escherichia-Aerobacter species, and the same was true of an unclean odor in milk set for cottage cheese.

In all, 3 species of Aerobacter and 9 species of Escherichia were identified as responsible for defects in the 11 samples examined.

A17. The Use of Streptococcus thermophilus in Ripening Milk for Swiss Cheese. WILLIAM C. FRAZIER, Bureau of Dairy Industry, United States Department of Agriculture, Washington, D. C.

The use of so-called "ripened" milk has long been recommended for the manufacture of Swiss cheese. A certain amount of ripeness has been considered desirable, but too much has been found to be harmful to the cheese. Natural ripening has been used for the most part, but this may result in an increase in gas-forming and other undesirable bacteria. Ripening of the milk with *Streptococcus lactis* has been used, but the control of the amount of ripening has been difficult and undesirable bacteria have often increased at the same time.

Experiments have shown that milk can be ripened by growth of *Streptococcus thermophilus* at 50°C. In practice one-fifth of the milk to be used for cheese is heated to 50°C. in the cheese kettle, inoculated with a culture of *Str. thermophilus*, held at that temperature for a definite period, and then cooled by the addition of the remaining milk to the kettle. This method places the ripening under control and eliminates the possibility of the growth of undesirable organisms during the ripening process. The *Str. thermophilus* organisms thus added apparently carry on most of their growth in the cheese kettle and are eliminated from action by the time of dipping or soon thereafter. Therefore the addition of the usual starter of *Str. thermophilus* is desirable, even when ripening with that organism is used.

Results in the laboratory and on a factory scale have shown that the quality of the cheese has been improved by ripening the milk with *Str. thermophilus* when that milk had a methylene blue reduction time of 6 hours or longer.

A18. Control of Anaerobic Sporeformers in Emmental Cheese Making. KARL J. DEMETER, South German Research Institute of Dairy Science, Weihenstephan-Muenchen, Bavaria. [Read by title.]

The anaerobic organisms were determined by 2 methods: the Burri method (shake-culture in glucose agar) and the Weinzirl method (so-called Sporogenes test). The first method indicates in general the sugar splitting group of anaerobic spore formers (butyric acid organisms) the second method the proteolytic group (Sporogenes and Putrificus types). In addition, samples of milk (mixed kettle-milk, flash-pasteurized at low temperature) and cheese (trier plugs taken after fermenting in the warm room) were inoculated in decreasing concentrations to get an idea of the quantitative aspect.

The frequency curve of the *butyric acid formers* in the kettle milk during 1931 showed an increase in March, then a drop followed by a maximum during the second half of May and all of June. The *proteolytic group* also showed some increase at the end of March, the maximum, however, was reached in December.

The testing of the cheeses for *quality* after the fermenting period in the warm room revealed an agreement with the respective results of the anaerobic kettle milk tests so far as the Burri method was concerned (gassy cheeses), whereas, in this line, no close relations were found to the results of the Weinzirl tests. In those cases, however, where *both* the Burri and Weinzirl tests of the kettle milk showed low content of anaerobic spore formers (September to November), the cheeses were almost blind.

The comparison of the anaerobic spore former tests of the kettle milk with the same tests obtained on the respective trier plugs about 6 weeks later showed quantitative agreement regarding the Weinzirl tests (proteolytic group) and discrepancies in the Burri tests (mostly higher values in the plugs than in the kettle milk). From this it may be concluded that in Emmental cheese the saccharolytic group of anaerobic spore formers is far more active than the proteolytic group.

Whereas the findings relative to cheese quality after the fermentation period held well for *groups* of cheeses, occasional cheeses showed exceptions to this rule. This was especially true when the amount of anae-

robic spore formers in the kettle milk was not very high. In this case it was difficult to draw, on the basis of the kettle milk tests, prospective conclusions as to how the cheese would behave during the fermentation period. It was further shown, however, that by clever use of *good* Emmental cheese starters even the strongest contaminations by butyric acid organisms could be paralyzed. Work is still continued.

A19. A Method for Detecting Chymase Production by Bacteria. Costantino Gorini, Agricultural High School, Milan, Italy. [Read by title.]

The usual methods for determining bacterial chymase (rennin or rennin-like enzyme) production are three: (1) Inoculate the organism into sterilized milk and observe if the milk curdles without the production of acid. This method is no longer adequate in all cases since I discovered the existence of bacteria which produce both chymase and acid in milk. (2) Filter the milk culture through a porous candle, add a small quantity of this sterile filtrate to sterilized milk and incubate at 37°C. I have observed that in this procedure some enzyme may be taken up by the porous candle. Moreover, both these methods have the disadvantage that heated milk is not as readily curdled by chymase as is unheated milk. (3) Add a small quantity of milk culture to fresh raw milk, incubate at 37°C, and examine at frequent intervals for a few hours. The period of observation must be so short that neither the bacteria present in the milk nor those added in the culture have time to act on the milk. But fresh milk may contain some chymase previously added to it in the udder by mammococcus and other bacteria (see below). I find these 3 methods insufficient for the detection of feeble chymase production.

I have devised a new method based on my old observation that *B. prodigiosum* and other bacteria produce chymase on agar cultures much better than in milk. The procedure is to take well-developed slant agar cultures, pour over them sterilized milk till the surface of the culture is covered over, mix the culture with the milk by means of a platinum needle and then incubate. The milk must be sterilized at not over 100°C. to cause as little change as possible and maintain its white color. This method has given positive results with many bacteria which appear to be inactive in ordinary milk cultures, e.g., many strains of *B. morgani*, *B. lipolyticum* of the udder, *Str. equi*, *Str. scarlatinae*, *B. typhi*. Blood agar is preferable. All these strains have curdled the milk poured over their slant cultures after an incubation of 8 to

30 days at 38°C. The clot was soft, alkaline or neutral and quite of a rennet-like nature (Rend. R. Acc. Lincei, 1932, **15**, 1000).

This confirms my previous results that chymase production is widespread among bacteria, especially parasitic forms, and is independent of the presence of milk and casein. So that this is not an adaptation function, but a normal function of the microbes.

A20. Temperature Uniformity in Bacteriological Incubators. M. W. Yale, Carl S. Pederson and Robert S. Breed, New York Agricultural Experiment Station, Geneva, New York.

Routine bacteriological analyses of milk by the standard agar plate method require incubators of large capacity that will hold uniform temperatures of 37°C. Variations in incubator temperatures are, at the present time, one of the greatest sources of error in agar plate counts made in routine milk control work because of the fact that 37°C. is near the maximum growing temperature for many types of bacteria found in milk.

Thirteen different incubators representing types sold by all the important manufacturers were studied. When packed full of plates with no space between piles or between piles and walls, the temperature variations ranged from 2.0° to 11.0°C. When the plates were piled 3 high and the piles spaced 1 inch apart, for 7 incubators tested, the temperature variations ranged from 2.5° to 8.0°C.

Water-jacketed incubators gave the most uniform temperatures of any of the types tested. In some types, the faulty placing of heating elements resulted in the over-heating of plates in certain places, while in others, the entrance of cool air through poorly located air inlets resulted in the underheating of plates. Too little ventilation helped to produce "spreaders" while too much ventilation caused too rapid drying of plates.

The fact that none of the incubators tested was capable of holding a uniform temperature of 37°C. indicates that definite specifications are needed. For the present, it is suggested that those incubators be regarded as satisfactory where the temperature in the chamber is maintained so that it does not fall below 35.5°C. nor rise above 37.5°C.

Where the temperature variation exceeds 2.0°, incubators may be used to a limited degree by selecting the space within the incubator where the temperature variation does not exceed the above limits. Many kinds of bacteriological work do not require as uniform incubation temperatures as does routine milk work. Incubators satisfactory for

the routine incubation of large numbers of petri plates would probably be satisfactory for other types of bacteriological work.

A21. Yeast, Its Manufacture and Use. CHARLES N. FREY, The Fleischmann Laboratories, New York.

The development of new processes based on the use of raw materials formerly not employed in the production of yeast necessitated radical changes in methods of production. A brief description of the new process is given. The uses of yeast in modern industry are described. Some recent applications, such as the feeding of irradiated yeast in the production of vitamin D milk, are discussed.

A22. Yeast Growth-Promoting Substances in White Sugars. H. H. HALL AND LAWRENCE H. JAMES, U. S. Bureau of Chemistry and Soils, Washington.

Difficulties encountered in the use of white sugars in the manufacture of certain types of food products led to a study of granulated sugar in a search for stimulating substances. One hundred nineteen samples of white sugar have been studied for their ability to support the multiplication of yeasts. Growth of the yeast was determined in inoculated 10 per cent solutions of the sugars by microscopic counts before and after incubation. Final yeast crops varied with different sugars and ranged from no increase to several hundred times the original.

Stimulating substances were partly or completely removed from the sugars by extraction with 80 per cent alcohol and recrystallization of the sugar. The alcoholic extracts obtained were concentrated, and when partly purified, each yielded 2 fractions. The addition of these fractions to 10 per cent solutions of the purified sugars afforded definite stimulation to yeast growth. Growth stimulating factors were not found in the extracts from any sugar which originally failed to support cell multiplication.

A23. Spoilage of Maple Products by Molds. CARL R. FELLERS, Massachusetts State College, Amherst.

Tub maple sugar contains 5 to 12 per cent moisture and actively supports the growth of certain molds, principally Aspergillus. Long continued growth of these molds produces off-flavors both in the sugar and in pure and blended table syrups made from the maple sugar.

Mold-infected maple sugar often becomes moist or semi-liquid due to the inverting action of invertase on a part of the sucrose. The invert

sugars thus formed are liquid and cause the so-called "sweating or runny" condition so often encountered. Both the growth of mold and the inversion of the sucrose are accelerated at higher temperatures. Normally these defects are most frequently encountered during warm weather or in sugars not held in cold storage. Because of non-crystallization, maple sugar softened or partly liquefied by the action of molds, cannot be used for the manufacture of solid maple candies or fondant.

Among the sources of contamination of maple sugar are wooden and tin containers and papers used for wrapping or covering. Mold spores do not survive the usual open-kettle sap concentrating process. Direct microscopic examination, using a Howard mold counting cell, enables the analyst to form an intelligent opinion of the mold content of the sugar and its probable suitability for storage or manufacture.

A24. Fermentation Flavors in Syrups. LAWRENCE H. JAMES AND H. H. HALL, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington.

Cane syrup imported from the West Indies as Barbados molasses is sold extensively in this country for its peculiar flavor. The syrup is made by a special process in which a portion of the sucrose is inverted by the addition of acid during the boiling process. When barrelled and shipped to the United States and stored for some time, a characteristic rum flavor develops. In an attempt to develop the same flavor in American products, Louisiana and Florida syrup were specially prepared and inoculated with microorganisms obtained from commercial Barbados syrup. After 20 weeks' incubation at room temperature flavors closely resembling those contained in the Barbados products developed. The fermentation is brought about by osmophilic yeasts with the production of alcohol, followed by a conversion of a large part of the alcohol to ethyl acetate esters, etc., by a group of organisms resembling *B. saccharobutyricus*.

A25. The Determination of Organic Acids Produced in Fermentations. O. L. OSBURN AND C. H. WERKMAN, Ames Field Station, U. S. Bureau of Chemistry and Soils and Bacteriology Section, Iowa Agricultural Experiment Station.

The partition method for the determination of organic acids as applied to fermentation liquors by Werkman, and Osburn and Werkman (Ind. Eng. Chem. (Analyt. Ed.) **2**, 302, 1930; **3**, 264, 1931) has been extended to include any possible mixture of formic, acetic, propionic and butyric

acids. The partition method has been compared with the Duclaux method, and it has been found by a combination of the two methods that the presence of acids other than the four mentioned can be detected.

The formic acid is determined separately and appropriate corrections made in the distillation and partition constants. A nomogram has been constructed from which the percentages of acetic, propionic and butyric acids may be read as determined by both methods. If no acids other than the four mentioned are present, the percentages of each acid as read from the chart will be the same for both methods. If some other acids are present the 2 methods give different percentages of each acid.

Analyses of over 20 mixtures have been made by both methods and the above findings hold without exception.

A26. Fermentation of Acetoacetic Acid and Pyruvic Acid by the Acetone-butanol Organism, Cl. acetobutylicum. W. H. PETERSON AND M. J. JOHNSON, University of Wisconsin, Madison.

By adding acetoacetic acid to actively fermenting cultures of *Cl. acetobutylicum*, and after a certain period of incubation, analyzing the culture for the added compound and the products formed, it was found that this acid had been fermented. A similar procedure with pyruvic acid showed fermentation of that compound also.

Acetoacetic acid was decarboxylated to form acetone. This change was effected both by actively reproducing cells and by cell suspensions. In a normal culture, the ability to ferment acetoacetic acid was greatest at the time of maximum production of solvents (acetone and butyl alcohol). In some of the best fermentations about 3.2 grams per liter of added acetoacetic acid were fermented in eight hours. Cell suspensions were most active when the medium was buffered with phosphate, pH 5.2 to 6.4, and decomposed about 1.5 grams per liter of acid in six hours.

Pyruvic acid (7.0 grams per liter) was fermented with the production mainly of acetic acid, acetone and acetyl methyl carbinol.

A27. The Use of Shredded Asbestos in Methane Fermentations. C. R. BREDEN AND A. M. BUSWELL, State Water Survey, Urbana, Ill.

This is a progress report of an investigation of the organisms responsible for methane fermentation. One of the difficulties connected with methane fermentations in the past has been the large amount of inoculating material necessary to start the fermentation. According to previous workers large amounts of decaying leaves, sewage sludge, canal

slime, etc., were necessary to insure successful inoculation. They reported that in the active culture most of the bacteria were concentrated on the solids and emphasized the fact that to inoculate a new fermentation it was necessary to transfer rather large amounts of this material and that the supernatant liquid was useless for this purpose.

During an experiment to determine the minimum inoculation required, it was found that when the suspended solids from a definite volume of liquor from a sewage digestion tank were collected on an asbestos mat and this mat used as inoculum a much better fermentation resulted than when an equal amount of solids without the asbestos was used. This suggested the idea that perhaps if the asbestos was present in the sterile medium it would not be necessary to use suspended solids as inoculating material. This was found to be true. By using a modification of Söhngen's medium with asbestos, it has been found possible to carry on the fermentation in test tubes sealed with vaseline and paraffin through many transfers using only a loop of the clear supernatant liquid from the previous culture as an inoculum. Using the same medium and technic without asbestos results in no fermentation.

A28. Development and Control of Microörganisms in Pulp and Paper Mill Systems. J. R. SANBORN, Research Division, International Paper Company, Glens Falls, N. Y.

The purpose of bacteriological investigation in the paper industry is, in general, two-fold: to control the development of undesirable microörganisms and to utilize those whose activities may be advantageous in paper-making processes. This report presents some of the microbiological problems involved in the control of the first group.

The invasion of pulp and paper mills by microörganisms and their subsequent development within the mill system usually result in economic loss and impaired quality, through the formation of mill slimes or the deterioration and discoloration of pulp and paper. Bacteriological analysis of process water and stock at significant stages in the paper-making operations, aided by special methods for filamentous fungi, furnish information relative to the efficiency of water purification methods, the distribution of microörganisms throughout the mill, and the points at which the pulp stream may receive large inoculations of organisms from growth accumulations.

Raw water supplies provide the main source of slime-forming organisms. In addition, the considerable degree of pollution often present in mill process water serves to emphasize the sanitary features of paper

products for personal and medical uses and of paper wraps or containers for foods.

The predominating slime-forming microörganisms vary from mill to mill and include bacteria, alga-like forms, and filamentous fungi of the mold type. Bacterial slimes, usually gelatinous, are formed by a flora quite typical of marine sources. Serious slime conditions, characterized by the formation of doughy or rubbery bodies, are caused by the Oidium-Monilia group. This group also includes certain yeast-like fungi of doubtful identity. Another troublesome type of slime is the result of the dense, tangled, masses of growth produced by *Aspergillus fumigatus* and other cellulose-destroying fungi. This general group includes members of the genera Chaetomium, Cladosporium, Acrostalagmus, Alternaria, Trichoderma, and Penicillium. The majority of these organisms are frequent inhabitants of pulp wood and are among the most active agents of pulp deterioration and discoloration. The control of microörganisms in the manufacture of paper is accomplished effectively through the aid of water purification processes, localized chemical treatments, mill sanitation, and special preservative methods.

A29. Bacteriological Studies on the Curing of Meat. F. W. Tanner and Florence Evans, University of Illinois, Urbana

Cured meats keep better than uncured meats which indicates that the curing solutions have a detrimental or selective action on microörganisms. Results of investigations carried out by de Freytag, Stadler, Forster, Weichel, and others, clearly indicate that curing, at least under the conditions which were used in their work, cannot be relied upon to destroy pathogenic bacteria which may be present in the meat. Since little or nothing is known about the effect of curing solutions on *Clostridium botulinum*, experiments have been in progress to determine the effect of the several constituents of curing solutions on *C. botulinum* and several other sporulating anaerobic bacteria. The salts used were sodium chloride, sodium nitrate, sodium nitrite, sugar and mixtures of these salts in amounts which approximated those used in actual practice. Experiments were made on media containing these salts and on meat itself. The meat was ground and the curing salt thoroughly mixed with it. It was then packed into deep tubes and overlayed with paraffin. The tubes were incubated at 37°C., in order to give the organisms every opportunity to develop. Salt was found to possess the greatest preserving effect.

Most of the strains of anaerobic bacteria used were unable to grow

in 10.5 per cent of sodium chloride in any of the substrates which were tested. This amount of salt also inhibited toxin formation by *C. botulinum*. The amount of salt which inhibited growth was usually much less than that required to inhibit toxin formation, although this was not always the case. Sodium nitrite was also found to exhibit an appreciable detrimental effect on the organisms used in these experiments. The amount required, however, was somewhat greater than that permitted in practical meat curing. Sodium nitrate in meat in culture tubes was found to have less effect than sodium nitrite, where again the amount required was considerably more than that permitted by food control regulations. Experiments were also made using mixtures of salt, sodium nitrate, sodium nitrite, and sugar. When these ingredients were combined in concentrations that could be used in practice, a greater germicidal effect was observed than when they were used separately. It is very doubtful whether the results secured in the laboratory in culture tubes are comparable with those secured under practical conditions in the meat cellar.

A30. Some New Heat Resistant, Acid Tolerant Organisms Causing Spoilage in Tomato Juice. ROBERT N. BERRY, American Can Company, Research Department, Maywood, Ill.

In the course of an investigation of off-flavor in commercially canned tomato juice, a new type of spoilage organism was found. The type species is named *Bacillus thermoacidurans*. It is described as follows:

Rod with truncate to rounding ends, 1.5–6.6 x 0.3–0.9 μ, average 3.0 x 0.6, occurring singly and in pairs, rarely in chains. No capsule. Motile by peritrichous flagella. Twenty-four-hour cultures gram-positive, older cultures both gram-positive and negative. Spores, terminal to subterminal, 1.2 x 2.0μ. Slow growth at 20°C., moderate to abundant at 37°, 55° and 62.5°C. Facultative anaerobe.

No visible growth in 72 hours at 37° and 55°C. in gelatin. Agar colonies: 1 to 5 mm. in diameter; circular, raised, glistening, smooth, opaque; no chromogenesis; no odor; butyrous consistency; medium unchanged. Nitrates not reduced; indole and H_2S not formed. Litmus milk: no change in 72 hours at 55°C. Moderate, dirty white, viscid growth on potato. Diastatic action strong. Krumwiede's triple sugar: acid throughout. Russell's double sugar: anaerobically, very slight acid, which disappeared on standing. Active growth occurs from pH 4.0 to 8.0 incl. Slight growth occurs occasionally as low as 3.0 and as high as 9.0.

Gas not produced in carbohydrates. Acid to brom thymol blue in arabinose, dextrose, d-levulose, d-galactose, mannose, saccharose, maltose. No acid is formed in xylose, rhamnose, lactose, raffinose, melibiose, inulin, dextrin, mannitol, glycerol.

Thermal death time. A concentration of 10 million spores per cubic centimeter gives a span, over one logarithmic cycle on semi-log paper, of 14.5°F. in tomato juice pH 4.5, and of 17°F. in phosphate buffered water pH 7.0, with an extrapolated "F" value (destruction time in minutes at 250°F.) of 0.33 minutes in tomato juice and 3.8 minutes in phosphate buffered water.

Habitat. Isolated from off-flavored canned tomato juice. While the spoilage is eventually of the "flat-sour" type, the off-flavor is observed some time before any change in pH occurs. The organism probably originates from the soil. It is not pathogenic to humans or guinea pigs when taken orally; nor to tomato plants or green or ripe tomatoes.

Two other organisms which also produce this peculiar type of spoilage and differ only in a few biochemical relationships, are named *Bacillus thermoacidurans* var. A and var. B, respectively.

From experimentally inoculated packs involving 2058 cans of tomato juice and 132 cans of other acid and non-acid fruits and vegetables, the following conclusions were drawn: (1) There was a decrease in pH in all incubated cans. This decrease was slight in uninoculated cans, but more pronounced in inoculated cans which became typically off-flavored. (2) A temperature of 37°C. appeared to be the optimum for the production of the off-flavor. (3) A heavy inoculation produced a higher incidence (24 per cent) of off-flavor samples than a light inoculation. (4) Processing for 10 minutes at 212°F. for a 10-ounce container gave 28 per cent more off-flavored samples than did 20 minutes at 212°F. (5) The organism produced the typical off-flavor in the juice made from Indiana tomatoes, but not from California tomatoes. (6) The organism does not appear to cause the off-flavor in some of the so-called acid products, while in the non-acid products its metabolism is that of a true "flat-sour" organism.

A31. The Processing of Tomato Juice. Carl S. Pederson, New York Agricultural Experiment Station, Geneva.

Considerable variation exists in the processing temperatures used in commercially bottled tomato juice. Previous work had shown that organisms found in spoiled tomato products were killed at a comparatively low temperature. Tests made in commercial plants have shown

that approximately 99 per cent of the organisms present on the tomatoes are killed by the pre-cook of 150° and that very few organisms survive a filling temperature of 180°. These are mainly spore formers that do not grow in tomato juice under normal conditions. The flavor of tomato juice and the number of organisms present depends upon the condition of tomatoes and the cleanliness in the plant. After the organisms present on the tomato have been killed they may settle and form a slight sediment in the juice.

A32. Frozen Foods from the Health Standpoint. LAWRENCE H. JAMES, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington.

Foods preserved by freezing in "consumer" packages include fish and shellfish, fruits, vegetables, dairy products, and meats. The additional handling and time required for preparation and packing into small containers increases the possibility of contamination of the raw materials with pathogenic microörganisms. The effects of freezing not only upon the texture and nature of the food products but also upon disease- and toxin-producing bacteria are important considerations in the handling and marketing of frozen foods. Investigators have shown that *E. typhi, S. suipestifer* and similar organisms are adversely affected by freezing, and may all be killed if frozen for a sufficient length of time. Further experimentation has shown that some foods which normally may carry toxin-producing anaerobes may still carry the spores after defrosting, but that under the usual methods of handling the anaerobes do not develop nor produce toxins. Pathogens are mostly destroyed by freezing. Frozen foods used promptly after defrosting do not constitute a health hazard to the consuming public.

A33. Destruction of Microörganisms at Various Freezing Temperatures. J. A. BERRY, Frozen Pack Laboratory, Bureau of Plant Industry, U. S. Department of Agriculture, Seattle, Washington.

The effect of storage at −2°C., −10°C., and −20°C. on the microbial content of frozen pack blackberries has been traced over a period of 13 months. In sealed tin cans destruction was rapid at −2°C., the "kill" amounting to over 99 per cent in 6 months, while in non-airtight containers growth took place, and the material was spoiled in 6 weeks. The destruction of microörganisms was less rapid at −10°C. than at −2°C., but was considerably greater than at −20°C., which showed a reduction

of only about 40 per cent at the end of the storage period. Similar results are being secured with strawberries and raspberries. The organisms concerned include representatives of the Cladosporium, Saccharomyces, Torula, Flavobacterium and bacillus genera. With cultures of these organisms in artificial culture media the effects of low temperatures were less striking, though a temperature of −10°C. was still more destructive than −20°C. It is believed that the CO_2 arising from the respiration of the fruit in the airtight containers at the higher temperatures acted as a germicide, the organisms remaining on blanched vegetables not showing corresponding decreases.

Ability to persist at low temperatures is not confined to spore-forming types. Viable cells of *Aerobacter aerogenes* have been found in spinach held for a year, and lactobacilli in peas held for 2 years at −10°C. Low temperature plus ice formation appears to be more destructive than cold alone. Growth of many organisms is inhibited at temperatures below −2°C., but species of Torula and Oidium have been found to grow slowly at −4°C. under some conditions.

A34. Lactobacilli in Frozen Pack Peas. J. A. BERRY, Frozen Pack Laboratory, Bureau of Plant Industry, U. S. Department of Agriculture, Seattle, Washington.

Microbiological analysis of some 40 samples of peas packed in various ways and held in frozen storage for 1 to 26 months at a storage temperature, in most cases, of 15°F. showed, after holding at ordinary room temperature for 2 to 7 days, a high content of both Gram-positive and Gram-negative rod forms, with spoilage of the material. Cultural methods yielded *Aerobacter aerogenes* and lactobacilli. The acidity ranged between 1 and 1.3 per cent, calculated as lactic acid, and pH values ranged between 4.2 and 4.6. Fifteen cultures of the lactobacilli isolated from this material were found to be identical with or closely related to *Lactobacillus cucumeris*. Since *Aerobacter aerogenes* and lactobacilli are not spore-formers, their ability to stand a temperature of 15°F. for over 2 years is noteworthy.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND COMPARATIVE PATHOLOGY

M1. Studies on the Meningococcus. LESLIE T. WEBSTER AND GEOFFREY W. RAKE, The Rockefeller Institute for Medical Research, New York.

A comparison is made of the morphological and biological properties of freshly isolated strains and old laboratory (stock) strains cultivated on artificial media over a period of years. Significant differences are found to exist. The standard monovalent sera made with stock strains are compared with monovalent sera prepared with freshly isolated strains. Such sera are found to differ quantitatively as regards agglutinins and qualitatively as regards precipitins, which latter are not found in the standard monovalent diagnostic sera. A method of agglutination, using the "fresh strain" sera, is described and compared with the more usual method over which it has certain advantages.

An attempt has been made to investigate the antigenic complex of the different types of meningococci. Three substances have been isolated, of which one is type-specific, another is a non-specific polysaccharide, and a third a group-specific nucleoprotein. A brief summary of the properties of these substances is given. The presence of the type-specific substance as a precipitinogen in the cerebrospinal fluid of cases of meningococcal meningitis has been demonstrated by the use of the monovalent sera prepared with freshly isolated strains.

M2. Studies on the Protective Substance in Antipneumococcus Serum. Study 21. Comparison of Certain Characteristics of the Water-insoluble Proteins of Antipneumococcus and of Normal Horse Serum. LLOYD D. FELTON, Harvard Medical School, Boston, Mass.

In order to investigate differences between the water-insoluble protein of normal horse serum and that of serum of horses immunized against pneumococci, comparisons were made on material prepared in the same way from these two sera.

Successive fractions of protein were made at 30, 36 and 50 per cent of saturation with ammonium sulfate. To establish the hydrogen ion concentration of minimum solubility, acid-base titrations were carried out, with the result that with immune serum, irrespective of types, that is, I, II, or III, the fraction insoluble at 30 per cent ammonium sulfate had a minimum solubility at pH 5.8 to 5.9; the 36 per cent fraction pH 6.1 to 6.2; and the 50 per cent fraction pH 6.8 to 7.0. On the other hand, these same fractions prepared from normal horse serum all had a pH of minimum solubility ranging from pH 6.0 to 6.2. At the point of minimum solubility of the normal, the protein in solution was 5 to 10 times that of the protein of immune serum. The percentage of protein of the different fractions compared to the total serum protein

was respectively, with 30 per cent, Type I immune 4.6 per cent, normal 3.8 per cent; with 36 per cent, Type I immune 7.3 per cent, normal 3.3 per cent; and with 50 per cent, Type I immune 3.0 per cent, normal 0.9 per cent. The highest protective potency in relation to the amount of protein was found in the fractions 36 and 50 per cent. The protein obtained by dissociating the S.S.-antibody complex was found to have a hydrogen ion concentration of minimum solubility at about pH 6.8.

M3. Immunology and Serology of the Typhus-Like Group of Diseases. L. F. BADGER, United States Public Health Service, Washington.

There is widely scattered throughout the world a rather large group of diseases commonly referred to as the typhus-like group of fevers. The viruses of many of the members of this group have been studied in laboratory animals. It has been shown that definite immunity is produced by the viruses studied. The immunological relation to each other of some of the viruses has been determined but such relation between the majority is as yet unknown.

The Weil-Felix reaction or the production of agglutinins to certain strains of *B. proteus* was first demonstrated with the virus of epidemic (European louse-borne) typhus. It is now known that the majority of the viruses of this group produce such agglutinins. These viruses produce agglutinins to *B. proteus* X_{19} in the sera of rabbits, monkeys, and white rats, as well as in the sera of humans. Complete agglutination in high titre is constantly produced by some of these viruses, while with others complete agglutination in significant titre is inconsistent. Of the two members of this group which are endemic in the United States, the virus of one, endemic typhus, constantly produces complete agglutination in significant titre, while that of the other, Rocky Mountain spotted fever, produces such agglutination inconstantly.

M4. Effect of Massive and Repeated Doses of Bacteriophage. N. W. LARKUM AND RUTH CORPRON, Michigan Department of Health.

Rabbits were given inoculations of staphylococcus bacteriophage as follows:

Daily injections of 2.0 cc. each intravenous, 25 doses.
Daily injections of 2.0 cc. each subcutaneous, 25 doses.
Single injection of 50 cc. intravenous.
Single injection of 50 cc. subcutaneous.

The reaction in the rabbit was followed by temperature and weight records and by numerous and frequent blood counts. The question of increased susceptibility to staphylococcus infection was investigated. After a brief rest period all rabbits were given massive intravenous injections of living staphylococci. The mortality as compared with controls shows a marked degree of protection. Some indication is given as to the relative efficiency of single large versus multiple small inoculations and as to the choice of routes of inoculation.

M5. Active Immunization Against Poliomyelitis. MAURICE BRODIE, Department of Bacteriology, McGill University, Montreal, Que.

The relationship between the infectivity of material used for active immunization against the virus of poliomyelitis and the resulting protection was investigated. Monkeys were inoculated intracutaneously with virus containing tissues of various degrees of infectivity. Previous workers had shown the results with virus attenuated by heat or germicides to be inconstant and unreliable and it was therefore decided to use living "virus" of low infectivity. Such was obtained from one of two sources; (1) from the cerebral cortex of animals killed at the height of paralysis (previously shown to be considerably less infective than the cord), (2) from the spinal cord of animals killed at various intervals after the height of paralysis. These are not strictly comparable as the first might be a highly infective virus in low concentration, while the second might be a greater concentration of a virus of low infectivity. In each case the infectivity of the tissue was determined by intracerebral inoculation. A first series of animals was inoculated with approximately one-half a skin infective dose of spinal cord removed at the height of paralysis. A second series received various parts of the cerebral cortex of the same cerebro-spinal axis, while a third set of monkeys was given approximately the same amount of cord tissue obtained from animals killed at various intervals after the height of paralysis.

The resulting protection, as tested by the neutralizing power of the serum, was found to be directly proportional to the infectivity of the inoculum, and only when a certain infectivity was reached did it produce immunity. This might be either a function of dose or of the degree of infectivity of the material.

Cord tissue of high infectivity was then used in combination with human convalescent serum. The virus-serum mixtures were given either combined, after incubation at 37°C., or separately, in which case

the virus was administered intracutaneously and the serum subcutaneously; and it was found that the amount of serum must be no more than is just sufficient to protect the animal from developing paralysis. This balance of serum and virus gave considerable immunity, while much less protection was afforded by a mixture containing an excess of serum.

In order to follow the onset and subsequent rate at which immunity developed, each of 2 animals was given approximately three-quarters of a skin infective dose intracutaneously. For the first 6 days there was no demonstrable immunity. Positive neutralizing power could be detected in the serum between the 6th and 10th days and reached its maximum by the 20th day after inoculation. Some 46 days later this maximum immunity was still unaltered.

(This research was made possible through a grant received from the Trustees of the Banting Research Foundation, Toronto, Canada.)

M6. Observations on the Elementary Bodies of Vaccinia. JAMES CRAIGIE, Connaught Laboratories, University of Toronto.

The elementary bodies of vaccinia represent the virus of vaccinia. They may be isolated from the skin of the rabbit and be washed free from the Seitz-filterable precipitable substance. Vaccinia virus has been transmitted in series through more than 20 rabbits employing washed elementary bodies as the inoculum throughout. Over 20 cc. of washed elementary body suspension sufficiently potent to give a confluent take in a 1:100,000 dilution and sufficiently dense to permit of macroscopic agglutination tests in dilutions of 1:20 to 1:50 may be obtained from one rabbit.

In previously vaccinated rabbits the inoculation of dead washed elementary bodies causes an increase of agglutinins commensurate with that produced by living elementary bodies. The early reaction following revaccination in adults has been elicited as readily with dead washed elementary bodies as with heated calf virus.

M7. Preservation of Purified Vaccinia Virus. C. A. BEHRENS AND W. W. FERGUSON, Purdue University, Lafayette, Indiana.

With the production of highly purified preparations of vaccinia virus the problem of preserving them presents itself.

These preparations are water-clear suspensions of the virus, free from tissue cells and extraneous matter, containing less than 0.06 per cent

of protein, and deteriorating when ordinary methods of preservation are applied.

It has been found that by the addition of 0.1 per cent gelatin or 1 per cent peptone most preparations have not deteriorated over a period of 7 months.

M8. Studies of Local Reactivity to the Bacterial Toxins. (A Preliminary Report.) AUGUSTUS WADSWORTH AND GRACE M. SICKLES, Division of Laboratories and Research, New York State Department of Health, Albany.

From the point of view that the phenomena of local reactivity in the experiments of Arthus, of Sanarelli, and of Shwartzman are fundamentally similar and are obviously manifestations of the action of foreign poisons as determined by different phases of tissue susceptibility, a series of experiments was undertaken with different pathogenic bacteria and their products. The results indicated some of the conditions of tissue susceptibility which were essential to the activity of bacterial toxin.

With the technic of Shwartzman, little or no local hemorrhagic response was elicited when the products of the diphtheria bacillus were injected either intradermally or intravenously. The observation was confirmed that hemorrhagic reactions may be obtained in skin areas of rabbits previously prepared with filtrates of known potency when products of streptococcus growth were injected intravenously. With the pneumococcus, reactions were obtained only in rabbits in which a dermal infection was induced and type-specific pneumococci were recovered at autopsy. Intravenous injections of bacterial filtrates of known reactivity, of 0.2 per cent agar, and, occasionally, of heavy suspensions of killed pneumococci induced these reactions.

From the results obtained in these experiments with the pneumococcus, the following technic was developed to determine the conditions under which bacterial products that were inactive became active. Rabbits sensitized to serum were injected intradermally with bacterial products, such as bile-lysed pneumococci, pneumococcus carbohydrate, and streptococcus toxin, or with live bacteria. Twenty-four hours later, 1 cc. of horse serum was injected intradermally in the same area, and 24 hours later an intravenous dose of meningococcus toxin was given. In some animals a marked hemorrhagic reaction appeared before the intravenous dose was given. In the remaining animals the intravenous inoculation was followed within 5 hours by marked hemorrhagic changes in the edematous area.

Control rabbits that had not been given toxin intradermally seldom developed hemorrhage but such reactions were occasionally obtained.

M9. Skin Response as a Measure of Immunization and Sensitization.
R. L. KAHN, Department of Bacteriology, University of Michigan, Ann Arbor.

In connection with studies on the relation between local and general response in animals following intracutaneous injections, a method has been utilized in this laboratory that holds promise of extending our knowledge of immunization and sensitization. This method is based on the capacity of the skin of albino rabbits to serve as a quantitative indicator, both in the realm of antitoxic immunity, exemplified by the Schick test, and in the realm of sensitization, exemplified by the Arthus phenomenon. If into the skin of albino rabbits, certain organic substances are injected at intervals and the skin response observed quantitatively, it is possible, on the one hand, to differentiate the substances possessing immunizing properties from those possessing sensitizing properties; and, on the other hand, to differentiate the immune state from the hypersensitive state in the animals. The following experimental plan gives illustrative examples.

Skin response in immunization. Into the hair-clipped skin of an albino rabbit are injected 0.1 cc. amounts of several high dilutions of diphtheria toxin and it is established that the highest dilution producing erythema is, say, 0.000003 M.L.D. Half a cubic centimeter of toxoid is injected intracutaneously in 3 doses. The resulting inflammatory response of the skin is noted. On redetermining the highest dilution of toxin that will produce skin erythema some weeks later, it will probably be found to be 0.00003 M.L.D. With repeated injections of toxoid, the skin may reach a stage of immunity, after several months, where it will give no response to intracutaneous injections of 5 M.L.D. of toxin or more. At the same time the skin response to the toxoid injections is negligible when compared with the marked responses to the early injections of this agent. In addition to the gradual increase of skin immunity to the toxin and toxoid, the rabbit serum shows a gradual increase in antitoxin. The quantitative determination of antitoxin is made by means of skin response in normal rabbits. The smallest amount of toxin that produces a definite skin reaction in rabbits following intracutaneous injection, is determined. This amount of toxin is mixed in series with varying dilutions of the immune rabbit serum and the mixtures injected intracutaneously in normal rabbits. The highest dilution of the immune

serum that neutralizes the toxin, thereby preventing a skin reaction, gives a measure of the amount of antitoxin in the serum.

Skin response in sensitization. Into the hair-clipped skin of an albino rabbit are injected 0.1 cc. amounts of a protein solution, such as human serum, in varying dilutions and it is established that only undiluted serum gives a mild skin response limited to slight thickening of the skin, the varying dilutions of the serum giving no skin response. A given amount, such as 0.5 cc. of human serum is injected intracutaneously in 3 doses and the resulting skin response is noted. About 2 weeks later, on reinjecting 0.1 cc. amounts of a series of dilutions of serum intracutaneously, it will probably be found that the highest dilution of serum producing skin erythema is 1:1000. With repeated injections of serum, the "skin sensitivity titer" may reach 1:100,000 or higher. The serum precipitin titer at the same time may be equally high. The frequent determinations of the precipitin titers parallel with the determinations of the skin sensitivity titers make possible the study of the quantitative relation between skin (local) response and serum (general) response in sensitized rabbits not only at a narrowly defined stage of sensitization but also during prolonged periods of sensitization.

Antitoxin immunity and protein sensitivity can be observed in the same animal. This is accomplished by injecting some protein with toxoid in an animal and observing the quantitative skin responses to the protein and toxin. The serum of this animal shows the presence of both, antitoxin and precipitins.

The relative lack of skin response in rabbits following intracutaneous injections of scarlet fever streptococcus toxin, brings to mind the fact that not all toxins follow the classical path of diphtheria toxin and raises questions of the nature of immune processes in these animals as well as of basic differences between the chemical constitution of toxins.

Application. The experimental plan outlined is being applied in this laboratory to the study of (1) antigenic specificity of proteins and protein split products, (2) some quantitative aspects of sensitization and desensitization, and (3) immunizing and sensitizing properties of some microörganisms and their products.

M10. The Preparation of Diphtheria Toxoid by Treatment of the Toxin with One Per Cent Formalin and Precipitation with Acetone. AUGUSTUS WADSWORTH, JAMES J. QUIGLEY AND GRETCHEN R. SICKLES, Division of Laboratories and Research, New York State Department of Health, Albany.

An earlier study having established that as soon as the detoxification of diphtheria toxoid is complete, the formalin may be eliminated by precipitation with acetone without apparent injury to the toxoid, further experiments were undertaken with larger quantities of formalin and shorter incubation at the ordinary incubator temperature. The results indicate that the detoxification is complete after from 4 to 5 days' exposure to 1 per cent formalin at 36°C. After 4, 5, and 10 days' exposure, the preparations were precipitated with acetone and restored to one-half the original volume with physiological salt solution. Toxicity and antigenic action were determined by the methods recommended by the National Institute of Health; in addition, tests of the antigenic activity, such as the titration of the antitoxin in the blood of immunized animals, were made, and flocculation values were determined. Slight but definite toxicity remained after the 4-day period of exposure which, however, had disappeared in the 5th and 10th-day preparations. The flocculation values were well maintained although the time of flocculation was greatly prolonged. The preparations incubated with 1 per cent formalin for 4 and 5 days apparently possessed antigenic properties equivalent to other preparations of toxoid which had been tested. The preparations incubated for 10 days showed a decrease in antigenic values.

The results thus demonstrate that diphtheria toxoid can be prepared by treatment of the toxin with large amounts of formalin, incubation at 36°C., and precipitation by acetone, without apparent loss of antigenic value, in 5 days, whereas the Ramon procedure requires from 4 to 6 weeks and special incubation at 39°C.

M11. The Purification of Diphtheria Toxoid. George F. Leonard and August Holm, Biological Laboratories, E. R. Squibb and Sons, New Brunswick, N. J.

The present work was undertaken in order to determine whether a suitable method could be developed for large scale production of a suitable purified toxoid. Such a method should meet the following conditions: It should not be complicated; it should give a satisfactory yield; more than half the protein should be eliminated; the purified toxin should respond to the flocculation test; it should be of such composition that sterilization could be affected by filtration; it should be stable for at least a year; and its antigenic value should be superior to average crude toxoid.

The various methods that have been proposed for the purification of

toxoid have been briefly reviewed, and those showing the most promise have been investigated.

Comparative experiments have been carried out with numerous samples of toxin and toxoid. All these have been prepared from 2 kinds of media, namely, the regular veal bouillon fermented with *B. coli*, and the semi-synthetic infusion-free medium of Kirkbride and others.

The results of the purification of diphtheria toxoid, prepared from the 2 types of media, when carried out by the different methods are given. Included in this comparison of results are: the flocculation units, milligrams of nitrogen per cubic centimeter, yield, and the purity of the purified products. The most feasible methods in our hands have been the precipitation by ammonium sulphate with subsequent dialysis, precipitation by acetone, and precipitation by alum. These comparative results outlined, give some of the advantages and disadvantages of the various methods proposed for the purification of diphtheria toxoid.

M12. The Production and Preservation of Diphtheria Toxoid. H. M. Powell, The Lilly Research Laboratories, Indianapolis, Ind.

Diphtheria toxoid of high potency has been regularly prepared from toxin produced in specially sterilized broth. Stability tests have been conducted which show that Merthiolate-preserved toxoid maintains immunizing and Lf qualities better than toxoid with phenoloid preservative.

M13. The Potency Testing of Vibrion septique Antitoxin. Ida A. Bengtson, United States Public Health Service.

The investigation concerns the establishment of a unit of measurement and methods of testing the potency of *Vibrion septique* antitoxin, one of the antitoxins used in the prophylaxis and treatment of gas gangrene. A study of 64 strains of *Vibrion septique* from various sources indicates that all produce toxins which are neutralized by the same antitoxin. As is true of *Cl. tetani* and certain other anaerobes there are several agglutination types (at least four) but all produce the same toxin.

The intravenous inoculation of rabbits and mice gave the most satisfactory results in tests to determine the most suitable laboratory animal and the best route of inoculation. Rabbits offer the advantage of ease of inoculation and rapid results. Mice are less sensitive but satisfactory tests may be made on this species also. The mouse offers the advantage of low cost and therefore more animals may be used. The rabbit inoculated by the intravenous route is highly susceptible to the action of

the toxin, symptoms and death occurring in as short a time as 2 to 3 minutes. Mice react less rapidly and are from 5 to 10 times less sensitive to the action of the toxin. For preliminary work the intracutaneous method of testing on guinea pigs may be used to advantage.

In histological studies the most striking observation has been that of the kidney lesions. These are not apparent in rabbits which die early but often occur in those which die after the first 24 hours.

Dried toxins and antitoxins to be used for standards have been prepared. With the purpose of establishing a unit of measurement which may be considered for international adoption comparative tests have been made with the provisional units of Great Britain, France and the Argentine Republic. A unit is being considered which has been determined by tests carried out on both rabbits and mice. Results obtained thus far indicate that more accurate titrations may be made on mice.

M14. A Method of Titrating Dysentery (Shiga) Antitoxin. E. G. D. MURRAY, Department of Bacteriology, McGill University, Montreal, Que.

The test depends on these observations: (1) The growth from an 18-hour agar culture of Shiga's bacillus heated at 56°C. for 30 minutes and dried to constant weight over H_2SO_4 or better still NaOH, in vacuo at 37°C., when inoculated subcutaneously into white rats gave a sharp Minimal Lethal Dose (M.L.D.). This showed no variation during 2 years. The dose is measured in milligrams per 100 grams of rat and kills in an average time of 65 to 70 hours (greatest variation 60 to 80 hours). (2) One-tenth M.L.D. always failed to kill and invariably conferred complete immunity on a rat, such that it was protected from one or more subcutaneous M.L.D. given 10 days later. (3) One-half M.L.D. killed the majority of rats in the usual time and 0.25 M.L.D. killed a smaller proportion of the rats with a delay up to 10 hours over the average period for 1 M.L.D. Rats which survived these doses were protected from 1 M.L.D. given 10 days later. (4) The subcutaneous injection of an amount of serum sufficient to protect a rat against 5 subcutaneous M.L.D.'s fails to protect against 1 subcutaneous M.L.D. given 10 days after the serum. (5) The reaction between the toxin and antitoxin is complete when mixed and incubated 30 minutes at 37°C. and no additional protection is afforded by the serum when the mixture is incubated for longer periods up to 2 hours.

The test is carried out by adding a selected constant volume of serum to each of a series of tubes containing the desired amount of a saline

suspension of dried bacteria, so arranged that 1 cc. of the mixture contains the number of M.L.D.'s it is desired to give to a 100-gram rat, together with the due proportion of serum. The mixture is incubated for not less than 30 minutes and then injected subcutaneously into rats weighing between 50 and 150 grams in a dose of 0.1 cc. to each 10 grams. In this way one series of rats receive 1 M.L.D. plus say 0.05 cc. of serum, and the other series each receive increasing amounts up to 7 or 8 M.L.D.'s plus 0.05 cc. of serum per 100 grams of rat, the increment in each series being 1 M.L.D. The volume of serum to use is determined in a preliminary experiment and is selected to give complete neutralization near the middle of the range of M.L.D's chosen. Ten days after receiving the mixture the rats which survive are given 1 M.L.D. subcutaneously.

The results of this test are easily interpreted, since rats which receive a dose of toxin exactly neutralized or over-neutralized will succumb to the second dose, rats which receive an excess of toxin amounting to 0.1 M.L.D. will be actively immunized and survive the second dose; rats which receive an excess of toxin amounting to 0.25 M.L.D. can be recognized because certain of them will die and others will survive the mixture of serum and toxin. Thus, it is claimed that the antitoxin value of the serum can be titrated to the amount equivalent to approximately 0.1 M.L.D. of toxin. The test requires 14 days to complete but, in compensation, it has the advantage of giving a close titration and a clear cut result.

That the test depends on a toxin-antitoxin reaction is shown by the following facts: (1) The suspension used as toxin did not contain any living bacteria. (2) The suspension produced extensive typical edematous and hemorrhagic lesions of the mucous membrane of the cecum and colon of rabbits when injected subcutaneously. (3) Absorption of the serum with living culture removed not only the agglutinating power of the serum completely, but the absorbed serum on titration contained only two-thirds or one-half of its original antitoxin content. This titration could be paralleled by diluting the original serum appropriately. (4) A suspension of Shiga's bacilli heated at 80°C. for 1 hour failed to kill rats and failed to immunize them, and failed to produce lesions in rabbits. (5) A suspension heated at 80°C. for 1 hour removed the agglutinating power of the serum completely but left the antitoxin content unaltered.

These observations also indicate that the agglutinating power of the serum plays no part in the protection it affords and therefore cannot be taken as an index of therapeutic value. Not all strains of Shiga's

bacillus are equally toxic and several strains have to be examined to select a suitable one for this test. With the strain used in these experiments the M.L.D. was 1 mgm. of dried bacilli per 100 grams of rat; also, living or dead culture would produce typical hemorrhagic lesions in the cecum and colon, blood-stained diarrhea and paralysis in rabbits with a subcutaneous dose of 2 mgm. of dry bacilli per kilogram of rabbit.

M15. The Isolation from the Rocky Mountain Wood Tick (Dermacentor andersoni) of Strains of Bact. tularense of Low Virulence for Guinea Pigs and Domestic Rabbits. GORDON E. DAVIS, CORNELIUS B. PHILIP, AND R. R. PARKER, United States Public Health Service, Rocky Mountain Spotted Fever Field Station, Hamilton, Montana.

Data are presented as evidence that strains of *Bacterium tularense* of low virulence occur in nature. Three such strains were isolated this year from specimens of the Rocky Mountain wood tick collected in western Montana. Guinea pigs infected with these strains by vaccination with spleen tissue died in 9 days. These results are comparable to those secured with a strain (Ohara-Haga coccus) of proved low virulence. Domestic rabbits infected with pure cultures (original isolations and first transfers) by cutaneous vaccination all survived, their infection being shown by subsequent agglutinin titers and prolonged resistance to 1,000,000 lethal doses of a highly virulent strain.

Guinea pigs and rabbits infected in the same manner with other recently isolated strains died in 4 to 6 and 3 to 5 days, respectively.

Because of its exquisite sensitiveness, the guinea pig is the animal of choice for demonstration of *Bact. tularense* infection. On the other hand, rabbits, because of their relatively greater resistance, are more satisfactory for tests of comparative virulence.

M16. A Study of Undulant Fever in New York State. RUTH GILBERT AND MARION B. COLEMAN, Division of Laboratories and Research, New York State Department of Health, Albany.

During 1930 and 1931, more cases of undulant fever were reported from New York than from any other state in the country. Furthermore, the findings obtained with specimens received for various types of serological tests suggest that scarcely one-tenth of the cases which occur are correctly diagnosed or reported. Apparently, only a small percentage of the physicians in rural districts, where the disease seems to be most prevalent, are familiar with the symptomatology of undulant

fever. In many instances, this diagnosis is not considered until agglutination with *Br. abortus* is obtained with a specimen of blood sent to a laboratory to be examined for evidence of another disease.

Nearly all of the strains of the abortus-melitensis group obtained from patients' blood or from cows' milk collected in various parts of New York state, which we have had an opportunity to study, have exhibited the characteristics generally attributed to the bovine type. These findings are in accord with epidemiological evidence which indicates that cattle or dairy products are the chief source of the incitant of undulant fever in this state.

M17. The Effect of Various Therapeutic Agents on Brucella abortus Infection in Guinea Pigs. C. M. CARPENTER AND R. A. BOAK, University of Rochester School of Medicine and Dentistry and Strong Memorial Hospital, N. Y.

Because of the numerous methods of treating undulant fever in man without satisfactory results, and since in many cases the symptoms subside spontaneously, it is obviously difficult to evaluate the various therapeutic procedures employed. Guinea pigs are quite uniformly susceptible to *Br. abortus* and much information can be obtained concerning the value of different chemicals, specific agents and irradiation from such therapeutic studies.

Seventy-five guinea pigs were injected subcutaneously with a culture isolated from man's blood, and 50 with a strain isolated from an abscessed bull's epididymis. Twenty were kept for controls, while groups of from 10 to 20 were treated by one of the following methods: (1) acriflavine intraperitoneally, (2) methyl-violet per os, (3) thionin per os, (4) immune serum intraperitoneally, (5) feeding autogenous vaccine, (6) injecting autogenous vaccine subcutaneously, (7) deep x-ray therapy, (8) artificially induced fevers. The dyes and the subcutaneous injection of the heat-killed vaccine failed to control the infection. Improvement was noted following the use of immune serum, vaccine feeding and artificially induced fever.

M18. Mucoid Encapsulated Streptococci with Particular Reference to the Differentiation of Streptococcus epidemicus, Mather's Streptococcus of Epidemic Influenza and Pneumococcus (Streptococcus) mucosus. ISADORE PILOT, University of Illinois, College of Medicine, Chicago.

Streptococcus mucosus is to be identified with the Pneumococcus group, and should be called *Pneumococcus mucosus*. Mucoid colonies of a non-hemolytic, green producing streptococcus in blood agar described in connection with epidemic influenza (Mather's coccus) differ from those of *Pneumococcus mucosus*. Mucoid encapsulated streptococci of the hemolytic type are identified as *Streptococcus epidemicus* which is responsible for milk borne epidemic septic sore throat. These encapsulated organisms of the Streptococcus-Pneumococcus group can be differentiated. Their relationship to epidemic disease is discussed.

M19. Cataphoretic Velocity and Virulence of Streptococci Isolated from Throats of Humans, from Raw Milk, Flies and Water During Epidemics of the Common Cold. E. C. ROSENOW, Mayo Foundation, Rochester, Minn.

The purpose of the study was to determine more closely, if possible, the etiologic significance and properties of streptococci associated with epidemic infections of the upper respiratory tract (the common cold, sore throat and bronchitis) and the cause of rise and fall of epidemic diseases, especially those involving the respiratory tract. The methods were similar to those used in experiments on elective localization and cataphoresis of streptococci as isolated in encephalitis and other diseases of the nervous system, and in arthritis, reported on previously. The glucose-brain broth medium in this study of epidemic disease, as in a study of non-epidemic diseases associated with streptococci, has served largely as a differential medium for the isolation of streptococci and in which characteristic properties of streptococci tend to be maintained. We have isolated streptococci having distinctive cataphoretic velocity and virulence from the throats of persons suffering from the usual autumnal infections of the upper respiratory tract, and at the same time from the raw milk and water supplied, and from flies. Evidence has been adduced which indicates that streptococci at certain times acquire peculiar virulence and other properties resulting in epidemic respiratory infections and that the simultaneous presence of streptococci having identical or similar physical properties and virulence as isolated from widely different sources is due to causes chiefly other than a spread by contact. The isolation from water of streptococci like those associated with epidemic infections of the respiratory tract may explain the reduction in incidence of these and other diseases as the result of purification of the water supply (Mills-Reinicke phenomenon).

M20. Experimental Meningitis in Guinea Pigs. S. E. BRANHAM AND R. D. LILLIE, The National Institute of Health, Washington.

In attempting to produce meningococcus meningitis experimentally it was found that guinea pigs are more susceptible to meningococci and their products than are other small laboratory animals. Clinical and histopathological meningitis was produced in 71.4 per cent of guinea pigs injected intracisternally with suspensions of sufficiently virulent strains of meningococci.

Essentially identical clinical and histopathological pictures were produced by similar injections in 91.6 per cent of guinea pigs given boiled suspensions, 91.3 per cent of guinea pigs given filtered suspensions, and 75 per cent of guinea pigs given filtered broth cultures of some strains of meningococci. Apparently this meningitis in guinea pigs, as has also been found in rabbits, is not necessarily an infection, but may be due to the toxic products of the meningococci.

Meningococci were recovered at autopsy from the cisternal fluid of 11 per cent of the guinea pigs given the living virulent cultures. The possibility that these recovered cocci represented survivors of the original inoculum must be considered, since all of the guinea pigs yielding them died within 24 hours after injection.

M21. Bacteriological Study of Acute Enteritis in Infants and Young Children. M. L. COOPER, Children's Hospital, Research Foundation, Cincinnati, Ohio.

This study was undertaken as an attempt to obtain further information regarding the bacteria involved in this acute infection, attacking mainly infants, with a mortality rate generally between 25 and 35 per cent.

Our series of patients studied numbered 46; were composed largely of infants; the mortality rate was 34.7 per cent; 5 were studied at necropsy and from the involved small intestine of each a green-producing streptococcus was isolated; this same streptocccus was shown to be present in the intestinal tracts of 65 per cent of these patients. Seventy-four per cent of these patients had a preceding upper respiratory infection and this streptococcus was found present in the intestinal tracts of 65 per cent of this group.

Ninety rabbits were injected intravenously with this microörganism; 70 per cent died; 92.4 per cent showed very marked gross pathology in the small intestine; these streptococci were recovered from the small intestine in 80 per cent of those cultured; 37 rabbits were injected with

these recovered streptococci, and 70 per cent of these showed the same gross pathology; and from these the same streptococci were re-isolated.

Six monkeys were injected with this streptococcus, all became sick; this microörganism was isolated from the stools of all 6 after injection while they had not been present before injection; 5 developed a diarrhea; 5 died; 5 showed very marked gross and microscopic pathology in the small intestine; the same streptococcus was isolated from the involved intestinal tracts of all 5 monkeys, these streptococci when reinjected into other animals reproduced a similar pathologic picture.

This streptococcus was also isolated from the blood of a fatal case and produced the same pathology in the intestinal tracts of a series of 17 rabbits, 3 monkeys and several mice. A similar streptococcus isolated from the throat of one of these patients with an acute upper respiratory infection produced the same lesions in the intestinal tracts of injected animals. It has been possible to protect our experimental animals with specific immune serum.

The microscopic pathologic findings were as follows in the involved intestinal tracts: extreme congestion of the mucosa; hemorrhages into the lymphoid nodules; polymorphonuclear and monocyte reaction in the mucosa; gram-positive diplococci present in the mucosa and submucosa.

M22. The Apparent Cause of an Infectious Enteritis of Very Young Lambs. HILDA HEMPL HELLER,[1] Division of Veterinary Science and Hooper Foundation for Medical Research, University of California.

There exists in California and apparently elsewhere a fatal epidemic enteritis of very young lambs, that is associated with lambing in sheds, and occurs in widely separated fulminating outbreaks. Diarrhea may or may not occur; death may supervene at the age of 1 or 2 days; there is severe toxemia and there are frequently nervous symptoms. The characteristic lesions are: severe hemorrhagic enteritis isolated in stretches but not in patches of the small intestine, necrosis of the intestinal epithelium, infiltration of the mesenteric lymph nodes, injection of the adrenals, congestion and atelectasis of large circumscribed portions of the lungs, and engorgement of the cerebral and other blood vessels. Dr. J. Howarth had previously demonstrated that intestinal content

[1] At present at Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh.

and mixed cultures therefrom confer the malady to lambs by feeding. This finding was confirmed.

The tissues and blood of the lambs are frequently sterile. Filtered extracts of the intestinal content contain a powerful poison that resists boiling and is not neutralized by botulinus antitoxins of types A, B, C and D; 0.25 cc. of such extract may kill an injected mouse in 2.5 hours. Mixed cultures and their filtrates from a large series of lambs contained this poison, which on intraperitoneal injection prostrated guinea pigs and caused death by respiratory failure in a few hours. Lesions of these guinea pigs resembled those of the lambs.

A long series of experiments in which lambs were fed treated intestinal content and cultures showed that it was highly improbable that an anaerobe causes this disease. Some of the colonies of colon bacilli from the intestine produced a poison of the identical effect as that of the intestinal extract and the mixed cultures; 4 cc. of filtrate from one colony culture killed a guinea pig in 2.5 hours. The loss of pathogenicity of the colon bacillus strains was phenomenally rapid; their colony variants were numerous. No more lambs were to be had when this organism was isolated.

This enteritis differs in lesions from English "lamb dysentery;" it resembles closely iso-colon bacillosis of calves (Christiansen). It partakes of the nature of food poisoning and of infection. It would seem to be caused by the formation of a poison in the milk in the intestine of lambs, by a virulent stage of a colon bacillus that is highly variable. Death is usually due to absorbed poison; septicemia may, however, occur.

M23. Host Adaptability of Treponema pallidum. Some Remarks on Koch's Postulates in Syphilis. E. E. Ecker, Institute of Pathology, Western Reserve University, Cleveland, Ohio.

In an extensive investigation covering 278 rabbits inoculated with 13 different strains (9 new and 4 old strains) of *Treponema pallidum* it was shown that the mean of incubation of the new strains varied from 30 to 44 days, while that of the older strains from 24.75 to 33.85 days. The mean of the maximum incubation time of the older strains almost coincided with the mean of the minimum incubation time of the younger strains. Each group appeared homogeneous and the incidence of takes of the younger strains was found to be 64.9 per cent, while that of the older strains 90.6 per cent, a difference of 25.7 per cent. These findings indicate adaptation of the organism to its new host and should be considered in all work in experimental syphilis.

Koch's postulates in this disease have not been completely fulfilled.

M24. The In Vivo Thermal Death Time of Treponema pallidum. R. A. Boak, C. M. Carpenter and S. L. Warren, University of Rochester School of Medicine and Dentistry and Strong Memorial Hospital, N. Y.

The in vitro thermal death time of *T. pallidum* has been determined to be: 5 hours at 39°C., 3 hours at 40°C., 2 hours at 41°C. and 1 hour at 41.5°C. This study is concerned with the effect of similar fever temperatures on the spirochaete in the bodies of rabbits with experimental syphilis. Because of the temperature gradients in the body it was necessary to use thermocouples to measure the temperature of the testes, syphilomata, and chancres. The rabbits' testes were immersed in constant hot water baths, while other rabbits were given a generalized fever by means of radiant heat. The length of time required to kill *T. pallidum* in the body by fever temperatures checked fairly well with the thermal death time obtained in vitro, but it was slightly longer at each temperature.

M25. Heredity as a Factor in the Resistance of Rats to Salmonella enteritidis. M. R. Irwin, Department of Genetics, University of Wisconsin, Madison.

Intraperitoneal injections of a fixed dose of a strain of *S. enteritidis* into a random mated stock of rats over a period of 2 years has produced no deviation from the mean mortality not attributable to errors of random sampling. During a 4-year period, an inbred strain has been uniformly highly susceptible to an equal dose of the pathogen.

Within the random mated race, the progeny of each of several successive generations of survivors, when subjected to the same dose of the organism, has shown a definite increase in the number of individuals resistant to the infection when compared with non-selected members of the same stock.

The progeny of reciprocal matings between survivors of resistant individuals and non-injected members of the susceptible inbred strain has shown an approach in percentage mortality to that of the resistant parental stock. The backcrossed and F_2 progeny showed a comparable degree of resistance.

There was no evidence that the increased resistance of the selected generations was due to an acquired immunity, active or passive. The results indicate that the resistance was in large part dependent upon a complex of genetic factors, some of which were at least partially dominant.

M26. A Study of the Infective Qualities Possessed by G Phase Cultures of Bacillus typhosus. PHILIP HADLEY AND HAIKAZ CARAPETIAN, University of Michigan, Ann Arbor.

The present study involved an attempt to ascertain whether there exists a virus-like stage in the life history of *B. typhosus*; and, if so, to study its effects on experimental animals.

Starting with a motile, S phase typhoid culture, the G phase was produced[1] by serial cultivation in infusion broth containing lithium chloride. This G culture was grown in infusion broth, filtered through a Berkefeld N candle and recovered from the filtrate. The recovered G culture was used for intraperitoneal inoculation into guinea pigs, the amounts being from 1 to 5 cc.

The inoculated animals almost invariably died within a period of from 20 to 93 days, or were killed for examination on the point of death. The gross pathological changes included loss of weight, rise in temperature to 40°C. or higher, and, at autopsy, enlargement of the spleen, congestion of the lungs and, in many animals, small hemorrhages in the brain. Splenic abscesses were frequently present.

Suspensions in salt solution or in broth of the spleen, liver and brain tissues of these animals, usually killed at the time of defervescence, were injected into fresh animals. In these a similar trend of reactions was set up. From these animals, in turn, the infection was transmitted, by use of both tissue suspensions and cultures from tissues, to other groups. The period elapsing to the time of death shortened and the febrile reactions increased as the passages were continued.

Cultivation in broth and on agar plates from the brain and other organs of animals of the earlier series resulted negatively in nearly every instance. Serial plate transfers, however, usually gave positive results in the form of G colonies on the second to fifth plate of transfer. From some animals no visible culture was obtained although further inoculations of the apparently sterile suspensions continued to transmit the infection without interruption. In all cases, at least in the earlier series of passages, the malady was transmitted from animal to animal as an *apparent* virus disease.

Following a sufficient number of passages through animals there were recovered cultures which appeared to be either intermediate between the G forms and the original culture or closely resembled the original culture. In 2 cases the recovered cultures were similar to the original

[1] In a separate study by Mr. Nathan Manow and the senior author.

morphologically, culturally, serologically and in sensitiveness to the homologous bacteriophage. They differed from the original strain in 3 respects. First, they showed a tendency to ferment glucose, maltose and mannite with the production of gas, thus simulating *B. paratyphosus B.* Second, they gave an earlier alkalining of litmus milk. Third, they were highly pathogenic for guinea pigs, the M.L.D. being 0.000,001 cc. while the M.L.D. of the original culture was from 1.5 to 2.0 cc. of broth culture.

On the other hand, 2 of these cultures agglutinated nearly to titer in an immune serum prepared against the original typhoid strain, but not significantly in serum immune to *B. paratyphosus B.* Moreover, serums prepared against these cultures agglutinated completely to titer the original typhoid antigen and also a Rawlings typhoid antigen; but they did not agglutinate significantly either parathyphosus A or B antigens. Other cultures of intermediate type also agglutinated significantly in known typhoid immune serum.

When brain or organ suspensions were filtered through Berkefeld N candles under low negative pressure and the filtrates cultivated by the serial plate method, the G phase cultures similar to those of origin were recovered. Up to the present time, however, a long series of cultivations in broth and on agar has not brought about a reversion to the original culture type.

Control observations on the temperatures of the animals were maintained throughout. Control tests involving cultivation by similar methods from the spleen, liver and brains of 6 normal guinea pigs failed to yield any visible growth. It should be added that, during the course of the experiments here reported, there was no epidemic disease in the stock and the number of deaths among uninoculated animals was insignificant.

The possible significance of these findings is briefly discussed but no conclusions are yet drawn from the experiments. It is pointed out, however, that the results are in some measure in harmony with observations of Friedberger on his alleged "krypt-antigenic virus" in typhoid, with the results of Bisceglie on his alleged virus of Asiatic cholera, with certain experiments of Sanarelli on virus-like forms from *B. typhosus* and *B. icteriodes*, and with much of the work of Calmette's group and others on the so-called ultravirus of tuberculosis.

M27. Bacillary Forms in Hemolytic Streptococcus Cultures. ALICE C. EVANS, National Institute of Health, Washington.

All investigators of hemolytic streptococci are familiar with occasional bacillary forms which are observed more frequently in freshly

isolated strains than after long artificial cultivation. A pleomorphic puerperal strain which had been cultivated artificially for 3 or 4 years recently came under my observation. Provided with certain conditions it grows in bacillary form with few coccoid cells, giving the general impression of the genus Bacillus rather than the genus Streptococcus. An attempt was made to provide conditions which would complete the metamorphosis so that multiplication might take place in bacillary form in all media. The attempt was unsuccessful, for the culture always returned to the streptococcus form when planted in blood broth and it always grew on agar in typical streptococcus colonies. Photographs illustrate the bacillary forms.

M28. Variation in Tubercle Bacilli in Pulmonary Tuberculosis. G. B. Reed, Christine E. Rice and J. H. Orr. Queen's University, Kingston, Canada.

Earlier papers have shown that the serum of rabbits immunized with established S strains of tubercle bacilli gives, with antigens prepared from S strains, approximately double the complement fixation obtained with the same serum and antigens prepared from the R strains. The serum of rabbits immunized with R strains gives approximately the same fixation with both S and R strains. Dividing the fixation with the S by the fixation with R antigens gives an *S/R index* in the former case of approximately 2 and in the latter of 1.

Complement fixation reactions on several hundred cases of pulmonary tuberculosis using both S and R antigens give indices from over 2 to less than 1. Those cases which gave indices over 1.75 were all regarded, by the clinicians in charge, as unfavorable; 50 per cent of the cases with indices less than 1.75 were given an unfavorable and 50 per cent a favorable prognosis. It is suggested that variation of the invading bacilli may be responsible and this thesis is supported, but not proven, by a study of sputum cultures.

M29. Variation in Cl. welchii. J. H. Orr and G. B. Reed, Queen's University, Kingston, Canada.

Plating out pure cultures of *Cl. welchii* on suitable media has given rise to the development of two distinctly different types of colonies, the one a smooth hemispherical mound with sharply defined margins, the "S," the other a flat granular colony with an irregular or flagellated margin, the "R." These two types present the usual morphological appearance and give the characteristic reactions of *Cl. welchii*. In

fluid media the S produces an even turbidity while in the case of the R, there is a heavy bottom growth with an almost clear supernatant. Cataphoresis and specific agglutination studies indicate a marked difference in the 2 types. The S is pathogenic for pigeons and rabbits and produces an active hemotoxin, the R shows little or no pathogenicity and a correspondingly small yield of hemotoxin.

M30. Studies on Variability of C. diphtheriae. LLOYD R. JONES, St. Louis University School of Medicine, St. Louis, Mo.

A typical culture of virulent *C. diphtheriae* grown in broth or on agar media at 45°C., irregularly gave rise to the development of several variant types, as follows: *Type A:* A temporary variant, differing from the parent type only in exhibiting markedly granular colonies quite irregular in contour which were unstable, readily reverting to the parent colony type. *Type B:* An hereditary variant, permanently avirulent but similar to the parent type in all other discernible characteristics. *Type C:* A temporary variant, regularly avirulent, differing markedly from the parent strain in colonial form by exhibiting a small transparent colony; practically devoid of fermentative ability and exhibiting pleomorphism in smear preparations ranging from small coccoid to elongated solid-staining and occasionally granular types. This variant was unstable, showing a tendency for partial reversion to the parent type colony under ordinary conditions of cultivation. *Type D:* An hereditary variant similar to the above type and derived in stable pure line strains through many consecutive re-isolations incident to persistent cultivation under the cultural conditions employed to incite the transformation. Similar Type D variants were obtained incident to cultivation of the parent culture in broth containing specific antibacterial serum, or phenol or lithium chloride. The properties which characterize this variant type, regardless of manner of derivation, have remained constant in cultures maintained over periods of 6 months to 2 years. All attempts to force the reversion from variant Type D back to the variant Type B or to the parent type by frequent transplantation in plain broth, or in broth containing heat-killed organisms of the parent type, or in broth containing specific variant antiserum, were unsuccessful.

Antiserum containing agglutinins for the parent type organism agglutinated the Type B variant and failed to agglutinate any of the Type D variant organisms. Type B variant antiserum agglutinated to a slight extent parent type organisms and did not agglutinate any of the

Type D variant organisms. Antiserum prepared with a particular Type D variant organism did not agglutinate either the parent type, or Type B variant organisms or other Type D organisms derived in dissimilar manner, however limited cross agglutination reactions occurred among various Type D variant strains derived upon different occasions from the parent culture by cultivation in broth at 45°C.

These results indicate antigenic dissimilarity between the virulent parent type organism and its avirulent variants exhibiting the small transparent colony, and further that antigenic differences noted among various strains of the latter occurred incident to using various methods for forcing the dissociation, whereas Type D variants derived by the same cultural technic were characterized by a perceptible degree of antigenic similarity.

M31. Acid-Fast Bacilli in the Blood of Tuberculous Patients. JOHN F. NORTON AND NORMA H. BROOM, Department of Health, Detroit, Michigan.

Cultures were made from the blood of 175 tuberculous patients. These were classified clinically as follows:

Pulmonary:	
Minimal	24
Moderately advanced	70
Advanced	53
Non-pulmonary	13
Unclassified	15

After investigation of various proposed technics, the following was determined upon. To 10 cc. of citrated blood, sterile distilled water was added and the mixture allowed to stand in the ice-box over night in order to obtain complete hemolysis of the red blood cells. In the morning, the tubes were centrifuged for one-half hour at high speed, and the sediment planted on Petragagni medium and on a modified Petroff medium. The sediment was divided so that 2 tubes of each medium were inoculated. The tubes were then sealed with paraffin and incubated. The tubes were examined weekly over a period of 90 days. In no instance was a true tubercle bacillus isolated. From 4 samples of blood, acid-fast organisms were obtained. These were of a highly chromogenic variety, grew extremely slowly on egg media, did not grow on glycerin agar or on plain agar, nor on Long's synthetic medium. They did not produce tuberculosis when injected into guinea pigs. Further studies are being made on these organisms.

Inability to cultivate tubercle bacilli from the blood does not necessarily mean that no tubercle bacilli are present. Studies have therefore been made to determine the minimal number of tubercle bacilli which can be discovered by the technic used.

M32. Organisms which Decompose the Specific Carbohydrates of Pneumococcus Types II and III. GRACE M. SICKLES AND MYRTLE SHAW, Division of Laboratories and Research, New York State Department of Health, Albany.

Several strains of soil organisms which decompose the specific polysaccharide of pneumococcus type III were isolated from decaying organic matter in different localities. One of the strains, unlike that previously described by Avery and Dubos, also utilizes agar. Experiments with the protective and curative effect in mice of the enzyme obtained from these strains confirmed, in general, the results of Avery and Dubos.

Several strains of an organism which decomposes the specific carbohydrate of pneumococcus type II have also been isolated. This organism, which differs markedly from the type-III carbohydrate-decomposing strains, is an obligate aerobe oval in form, which in some preparations resembles a yeast. It grows readily in a medium containing ammonium sulfate, potassium phosphate, and type-II carbohydrate but with difficulty or not at all in the usual liquid nutrient media. No growth is obtained on solid nutrient medium and growth on a medium composed of agar and the mineral medium containing type-II carbohydrate is very slow. Colonies on solid medium and the bacterial sediment in liquid medium have a marked pinkish color. A 24-hour culture in mineral medium containing type-II carbohydrate withstood heating for ten minutes at 52°C. but did not survive at 54°C.

No protection tests in animals have been possible since all attempts to obtain a soluble enzyme have been unsuccessful. No activity has been demonstrated against the specific polysaccharides of pneumococcus types I and III by the cells themselves.

M33. A Study of Typhoid Agglutinins and Conditioned Reflex. NICHOLAS KOPELOFF, LENORE M. KOPELOFF AND MARY E. RANEY, Department of Bacteriology, Psychiatric Institute and Hospital, N. Y.

Since the possible rôle of the nervous system in the production of antibodies is still obscure, an attempt was made to investigate typhoid

agglutinin production in human beings who were subjected to Pavlov's conditioned reflex procedure.

Neurotic and psychotic patients in our hospital were conditioned for 21 consecutive days, by the same operator, at exactly the same hour and under exactly the same environmental conditions by means of a thermal stimulus, i.e., an ice-cube applied to the right cheek-bone followed by a subcutaneous injection of a small dose of typhoid vaccine. A rest period of about 2 weeks followed. Titers were measured weekly and when they fell consistently the subjects were given the thermal and tactile stimuli: ice-cube and needle prick, with or without saline in the original setting. Four such experiments were performed and 65 subjects were studied.

It was clearly brought out that there are important psychologic factors which influenced the reading of agglutinin titers. These were minimized by an objective procedure which has been described in detail.

The final conclusion was that empirically and statistically there was no evidence that a conditioned reflex was established nor that if it had been, it exercised any influence on the typhoid agglutinin titer when injected subjects were compared with appropriate controls.

M34. The Detoxification of Pollen Antigens. GEORGE E. ROCKWELL, Department of Bacteriology, University of Cincinnati.

Desensitization of hay fever subjects by hypodermic injections of pollen extract is not entirely satisfactory, because it is often attended with mild-to-severe reactions. Hence, the dosage cannot be increased very rapidly. If such a pollen antigen could be treated so that it would not give these reactions, and still be potent for the prevention of hay fever, it would be a distinct improvement. This we have attempted to do.

Ragweed pollen extract was oxidized by treatment with hydrogen peroxide. The hydrogen peroxide was then removed by the use of platinized asbestos. This oxidized pollen extract gave little or no skin reaction when sensitive individuals were tested.

Such oxidized ragweed pollen antigen was used in the treatment of 3 hay fever subjects. There were no reactions, and hence the dosage could be increased much more rapidly than it could possibly have been increased if regular ragweed pollen extract had been used. The clinical results were very satisfactory.

APPLICATION OF STATISTICS TO PROBLEMS IN BACTERIOLOGY

I. A MEANS OF DETERMINING BACTERIAL POPULATION BY THE DILUTION METHOD

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Received for publication, April 2, 1932

INTRODUCTION

Some time ago the authors had occasion to study the effect of bacteria on the curing of meat. As a part of the work it was necessary to determine the number of bacteria present in the pickle at different stages of the cure. When plates were made of the pickle in the usual manner, surprisingly low counts were obtained, as compared with the number estimated by direct microscopic observations of wet preparations. While the latter indicated from 500,000 to 1,000,000 organisms per cubic centimeter, the plate count obtained from the same material was 10,000 or less. The low values obtained with plating were at first attributed to an unfavorable medium; consequently a solid medium was made by adding agar to clarified pickle. The counts obtained with the use of this medium were approximately the same as those obtained with use of standard beef extract agar. These results led us to believe that a considerable portion of the usual flora of curing pickle could not develop on solid media. Attempts were then made to determine the number of bacteria present by means of the direct count. For this purpose a modification of the Breed and Brew (1925) method was used. This was not satisfactory because of the high salt content of the pickle. The salt not only brought about a precipitation of the dye, but also, in crystallizing, obscured many of the organisms. Furthermore, the salt prevented proper fixation of bacteria on the slide, so that

attempts to remove the salt by washing resulted in the removal of most of the bacteria. Because of the great variety of organisms present, many of which were actively motile, and because of the presence of débris, methods making use of a counting chamber could not be used.

These difficulties forced us to consider the dilution method as a means of evaluating the bacterial population.

We were at once confronted with the following problems: (1) calculation of the number of bacteria present from the number of tubes showing growth in the various dilutions, (2) evaluation of the accuracy of the data thus obtained, and (3), determination of the variation in accuracy with the number of tubes used in each dilution. A careful review of the literature was made in an attempt to find answers to the questions involved. We found that many investigators had advanced equations that would enable us to calculate the most probable number of organisms from the number of tubes that showed growth, and some had published tables to aid in the calculation. We were, however, unable to find any satisfactory solution of the problem of variation in accuracy with the number of tubes used in each dilution, or, what would have been more desirable, a method for the determination of the number of tubes that must be used to get a specified accuracy. We therefore deemed it necessary to reconsider the entire problem. This is the first of a series of articles dealing with the various problems involved in the dilution method of determining a bacterial population.

HISTORY

The use of dilution methods in bacteriology dates back to the early days of the science. About 1875 Pasteur obtained pure cultures of bacteria by diluting the original inoculum during several successive transfers to a suitable culture medium. Later Miquel, Brefeld, and Lister (Kolle, Kraus and Uhlenhuth (1930)) obtained pure cultures by inoculating small amounts of diluted bacterial suspension into a series of tubes of medium.

For many years bacteriologists have been using dilution methods to give some idea of the number of organisms in the material

examined. This method consists in diluting the material to be examined, usually in powers of 10, and inoculating equal volumes of the diluted material into liquid media. If growth occurs from the inoculation of 1 cc. of a 1:100 dilution and not from a 1:1000 dilution, the number of organisms present in the original material is said to be between 100 and 1000 per cubic centimeter. On the basis of chance, however, it might be possible to have only 50 organisms per cubic centimeter, or more than 1000 per cubic centimeter in the original material and still get growth from the 1:100 dilution and not above that in a single test. A presumptive coli test, utilizing varying amounts of inoculum, has been used in a similar way in the bacteriological analysis of water to give an approximate idea of the quality of the water.

This was essentially the basis of the method introduced by Phelps (1908) to estimate the *B. coli* content of water from presumptive test data. In his method it is assumed that the reciprocal of the highest dilution which shows growth represents the most probable number of organisms present. His method was adopted by a Committee on Standard Methods of Water Analysis of the American Public Health Association (1920). In case "skips" occurred, that is, a positive presumptive test from a dilution higher than one which was negative, the result taken was the reciprocal of the dilution next higher than the smallest one giving a positive test.

A more accurate method of interpreting dilution data has been supplied by McCrady (1915). In developing his equations he begins with the proposition that there is only one organism for each 100 cc. in the sample. He stated that this one organism must obviously be contained in one of the 1 cc. volumes and that the probability of not getting the organism when a single cubic centimeter is removed from the 100 cc. volume is 0.99. The following quotation from his article (p. 185) shows how he has developed his equation.

> Now suppose 2 *B. coli* are in the sample. The probability of each organism's not being contained in the 1 cc. withdrawn for the fermentation test has been shown to be (0.99). Then, by the principle just il-

lustrated, the probability of neither organism's appearing in this 1 cc. is equal to the product of the separate probabilities, or (0.99) (0.99) = 0.9801. And if a great number of such samples were examined, about 98.01 per cent of the results would be "0/1 in 1 cc."

In general if V represents the number of volumes in the sample, and x the number of *B. coli* in the sample, and one volume is withdrawn, the probability that this volume will contain no *B. coli* is given by

$$\left[\frac{V-1}{V}\right]^x$$

Thus when 1 cc. of the sample is withdrawn for the test, V becomes 100 and the formula becomes $\left[\frac{99}{100}\right]^x$. When a 10 cc. quantity is withdrawn, V becomes 10 (there are ten 10 cc. volumes in the sample), and the formula becomes 0.9^x.

With this reasoning as a foundation for his later work, McCrady developed the equations from which it is possible to calculate the most probable number of organisms per cubic centimeter from data obtained by inoculating a series of tubes with the same dilution or from several series of tubes inoculated with several different dilutions. For the special case where a series of tubes are inoculated with 10 cc., a second with 1 cc., and a third with 0.1 cc., the following formula is given:

$$(p+q)(\log 0.9)+(r+s)(\log 0.99)+(t+u)(\log 0.999)=\frac{p(\log 0.9)}{1-0.9^x}+\frac{r(\log 0.99)}{1-0.99^x}+\frac{t(\log 0.999)}{1-0.999^x}$$

In this equation, p, r, and t represent the number of tubes showing growth in the different series and q, s, and u represent the number of tubes showing no growth in the corresponding series of dilutions. To simplify the use of the method, McCrady (1918) has solved the equation for all possible combinations for several special cases, including those when 5 and 10 tubes are used in each dilution. These solutions have been put into tables so that the method may be used without tedious calculation.

While McCrady's tables and equations may be regarded as solving the problem of calculating, from dilution data, the most probable number of bacteria present, they do not offer a solution

to the more general problem of variation in accuracy with the number of tubes used. In attempting to solve this problem with McCrady's equations, we find that the mathematics become very cumbersome. McCrady's equations are each limited to a special case, so that to solve them one must resort to rather tedious calculation. A more general solution whereby all cases could be evaluated from a single equation and a single table is very desirable. We have accordingly approached the problem from a different angle and have worked out tables somewhat analogous to those of McCrady.

On the basis of minor assumptions, Wolman and Weaver (1917) have simplified McCrady's formula. Their equations are convenient for the calculation of the most probable number of bacteria, but they do not enable one to evaluate the accuracy of the data.

Various methods of interpreting dilution data have been considered in a series of articles by Wells (1918; 1919; 1921), and in an article by Wells and Wells (1922). Their treatment does not lead to results that can be used in the general solution of the problem.

The methods advocated by Wells have been objected to by several investigators. Notable among these is Cairns (1918).

More general considerations of the dilution method have been contributed by Stein (1922), Greenwood and Yule (1917), Fisher (1925), and Reed (1925). All of these men have shown that the number of tubes showing no growth when inoculated with a fixed quantity of a single dilution is equal to e^{-ax}, where x represents the number of organisms per cubic centimeter in that dilution, and a represents the volume of the dilution used for the inoculation.

Stein not only made use of this exponential relationship to interpret dilution data, but he attempted to simplify its application by graphical means. He also showed by graphical means how the accuracy of the method varied with the number of tubes used. These considerations were, however, limited to a few special cases.

Greenwood and Yule considered not only the special case of a

single dilution inoculated into a series of tubes but also the general case of several dilutions inoculated into a series of tubes. Although they give the equation from which the most probable number of organisms can be calculated from experimental data involving several dilutions and several tubes in each, they have made no attempt to simplify the solution of this general case. Since the general equation is rather involved, it is necessary to simplify the solution by means of tables in order to make it applicable for practical use. Greenwood and Yule show how the accuracy of dilution data can be evaluated. Here again, simplification is needed for general application.

Their equation for the general case is as follows:

$$(a_1n_1 + a_2n_2 + \ldots\ldots a_nn_n) = \frac{a_1m_1}{1 - e^{-a_1x}}e^{-a_1x} + \frac{a_2m_2}{1 - e^{-a_2x}}e^{-a_2x} \ldots\ldots \frac{a_nm_n}{1 - e^{-a_nx}}e^{-a_nx}$$

In this equation x is the most probable number of bacteria per cubic centimeter that will give n_1 negative and m_1 positive results when N_1 tubes are inoculated with a_1 cc. each, and will give n_2 negative and m_2 positive results when N_2 tubes are inoculated with a_2 cc. each, etc.

Reed's contribution is very helpful in the special cases where one tube in each of several dilutions or where several tubes of the same dilution are used, but his solution is not extended to the more general problem of several tubes in each of several dliutions.

The dilution method has been used by Cunningham (1915), by Cutler (1919a), and by Cutler, Crump, and Sandon (1922) to evaluate the number of protozoa in soil. In the first two of these publications the dilution data were interpreted by the method of Phelps (1908), while Cutler, Crump, and Sandon used a table calculated by Fisher (1925). This table by Fisher is useful only for a very special case, so that it is not of any material value in general application. Fisher's table appears to be calculated from the exponential function e^{-x}.

A theoretical consideration of the dilution method has also been contributed by Clark (1927), who applied the method to determine the numbers of bacteriophage in a suspension.

After a careful review of the literature on the subject we still

feel it is necessary to reconsider the entire problem. General as well as special equations should be developed that are based on the reasoning used by Stein (1922), Greenwood and Yule (1917), Fisher (1925), and Reed (1925). These equations should be solved for all the special cases that are in common use, and the solutions should be arranged in tabular form, as McCrady did for his equations. Furthermore, tables should be made available which will aid in the solution of any special case not in common use.

A more detailed consideration of the accuracy of the method is needed. The calculation of the accuracy should be simplified so that anyone using the dilution method will be able to determine, to a reasonable degree, the limits of accuracy of his data. This must be made simple enough so that it can be applied generally.

The mathematics involved in the dilution method should also be applied to other problems in bacteriology, such as the determination of the percentage of insects that may be infected with certain viruses, or the interpretation of data obtained when a series of animals are injected with a single dilution or several dilutions of a given pathogenic bacterium or virus. It is also desirable to investigate the effect produced on dilution data if we accept the theory that single cells cannot develop.

A consideration of these and other probability problems will be published in a series of articles on the subject. It has been deemed necessary to verify by experimental data some of the mathematical considerations, and data thus obtained will also be presented in this series.

This first paper is confined to the development of equations to be used for the evaluation of bacterial populations by the dilution method. Tables are included which aid in the solution of these equations, as well as special solutions which simplify their general application.

THEORY

In order to determine the number of bacteria in a sample of liquid material by the dilution method it is necessary to dilute

the material to such an extent that when a sample is removed, bacteria may or may not be present. The problem at hand, then, is to determine the probability of getting bacteria in a certain sample. This probability will depend upon the number of organisms present.

A number of earlier investigators have shown how this probability is related to the number of organisms present. It is believed desirable, however, to develop these relationships from fundamental reasoning so that it will be possible for those not familiar with Poisson's Series to understand the derivation of the equations without having to make a special study of the mathematics on which the Poisson's Series is based. An understanding of the derivation of the equations is necessary for their proper application.

There are several ways in which the bacterial population can be determined by the dilution method. One may determine the number present by inoculating a large number of tubes of media with an equal volume of the sample to be tested, and determine the number present by the percentage of tubes that show growth. Or one may inoculate a series of tubes of media with a given volume, another set with a smaller volume, and a third set with a still smaller volume, and then determine the number of organisms present by the number of tubes showing growth in the different series.

We are interested, therefore, in several cases: (1) the probability of getting growth in a single tube, when it is inoculated with a definite volume of the sample; (2) the probability of getting a certain number showing growth out of a series of tubes, all of which are inoculated with the same volume of a given sample; (3) the probability of getting a certain combination of tubes showing growth out of several series of tubes when the tubes in each series are inoculated with different sized samples.

Case I we can designate as a single tube of a single dilution; case II, as several tubes of a single dilution; and case III, as several tubes of each of several dilutions.

CASE I

To calculate the probability that an organism will or will not be contained in a certain sample, let us assume that a large volume (N cc.) of the material to be sampled be at hand, and that in this material there are x organisms per cubic centimeter. Let us assume further, that the volume of an organism is v cc. (unit volume), that all organisms have the same volume, and that the water present be divided up into particles of the same unit volume as a bacterium. If we imagine now that we remove one of these particles, and let P equal the probability that our selection will not be an organism, then

$$P = \frac{\frac{N}{v} - Nx}{\frac{N}{v}}$$

This will be true because $\frac{N}{v}$ represents the total number of particles in a volume N. Nx is the number which are bacteria, and $\frac{N}{v} - Nx$, the number which are water. The probability Q that the selection will be an organism is $\frac{Nx}{\frac{N}{v}}$. These expressions can be simplified to $P = 1 - vx$; $Q = vx$.

Let us assume that enough of these small particles are removed so that the aggregate is 1 cc. The total probability will be expressed by the binomial $[(1-vx) + vx]^{\frac{1}{v}}$. The first term of the binomial will be $(1 - vx)^{\frac{1}{v}}$, which represents the probability that no organisms will be contained in a 1 cc. sample.

$$P = (1 - vx)^{\frac{1}{v}}.$$

This can be simplified as follows:

$$\ln P = -x - \frac{vx^2}{2} - \frac{v^2x^3}{3} - \frac{v^3x^4}{4} \cdots\cdots$$

Since v is very small, the second and following terms will be very small in comparison with the first term as long as relatively small values of x are considered.

Therefore

$$\ln P = -x$$

or

$$P = e^{-x} \tag{1}$$

If instead of taking out 1 cc., we remove a cc., then

$$P = (1 - vx)^{\frac{a}{v}}$$

$$\ln P = a\left[-x - \frac{vx^2}{2} - \frac{v^2x^3}{3} \cdots\right]$$

$$= -ax$$

or

$$P = e^{-ax} \tag{2}$$

and

$$Q = 1 - e^{-ax} \tag{3}$$

CASE II

If several tubes are to be inoculated with the same dilution the number of tubes which show growth will depend upon the number of organisms present and upon the element of chance.

If n tubes are inoculated, the total probability will be expressed by the binomial $[e^{-ax} + (1 - e^{-ax})]^n$. By expanding this binomial, the following series is obtained:

$$(e^{-ax})^n + n(e^{-ax})^{n-1}(1 - e^{-ax}) + \frac{n(n-1)}{2!}(e^{-ax})^{n-2}(1 - e^{-ax})^2 \cdots$$

The first term of this series gives an expression for the probability that none of the tubes will show growth, the second term for the probability that only one of the tubes will show growth, etc.

If we let p equal the number of tubes that show growth and q the number that show no growth, in which case $p + q = n$, the series becomes:

$$(e^{-ax})^q + n(e^{-ax})^{q'}(1 - e^{-ax})^{p'} + \frac{n(n-1)}{2!}(e^{-ax})^{q''}(1 - e^{-ax})^{p''} \ldots\ldots$$

If we let P be the probability, then the following equation will hold for any combination of p and q:

$$P = \frac{(p+q)!}{p!\,q!}(e^{-ax})^q(1 - e^{-ax})^p \tag{4}$$

If p, q, and a are kept constant in this equation, P will vary only with x. From the equation and the nature of the problem, it is evident that there must be a maximum value of P corresponding to some particular value of x. This optimum value of x can be found by differentiating equation (4), which gives the following:

$$\frac{dP}{dx}\frac{1}{P} = -qa + \frac{pa\,e^{-ax}}{1 - e^{-ax}}$$

By putting the derivative equal to 0, it is possible to find the value of x that corresponds to the maximum value of P. If we let $\bar{x}$ be the optimum or most probable value of x, its value may be found by substituting $\bar{x}$ for x in the above equation, when the derivative is placed equal to 0. The resulting equation simplifies to

$$\bar{x} = \frac{1}{a}\ln\frac{n}{q} \tag{5}$$

In the case of several tubes in a single dilution, the most probable number of organisms per cubic centimeter is given by the natural logarithm of the ratio of the total number of tubes to those that show no growth. Changing from base e to base 10, the expression becomes

$$\bar{x} = \frac{2.3026}{a}\log\frac{n}{q} \tag{6}$$

In deriving this equation, it has been assumed that in bacterial suspensions, all values of x are equally likely to occur between

0 and a number determined by the maximum bacterial population that can occur in a sample. By means of this formula it is easy to calculate the most probable number of organisms present in a sample from the number of tubes that show no growth. This can also be obtained by means of equation (5) and table 1 (appendix). From equation (5), we have

$$\frac{q}{n} = e^{-ax}$$

The function e^{-ax} then gives the fraction of tubes which show no growth. By means of a table showing values of e^{-x} for different values of x, it is possible to determine the most probable number of organisms present corresponding to any value of $\frac{q}{n}$. Such tables have been calculated by other investigators (L. von Bortkewitsch (1898) and H. E. Soper (1915)). The table of Bortkewitsch is carried out to only four places and that of Soper to six places. Whereas these tables may be adequate for the calculation of the most probable number from dilution data, we found that they did not suffice for the calculation of the frequency distribution of experimental values that might be obtained on a single suspension. Such calculations are essential in order to show a convenient relationship between the accuracy of the data and the number of tubes used in each dilution. For this reason we found it necessary to calculate these tables to nine decimal places. They are included in the appendix. Values of the logarithm of $(1 - e^{-x})$ are included in this table to simplify the solution of other problems which will be discussed later.

Examples: Suppose that in an experiment 0.100 of the tubes inoculated with a cc. each showed no growth, then from the table we find that the value of x corresponding to $e^{-x} = 0.100$ is 2.30. This number is therefore the most probable number of organisms present in the size sample used for the inoculation.

By means of this table it is also possible to determine the percentage of tubes showing growth which are inoculated with different sized samples. Let us assume that a liquid contained 0.650 organism per cubic centimeter, or 65 organisms in a 100 cc.

sample. In this case there will be 0.0065 organism in 0.01 cc., 0.065 organism in 0.1 cc., and 0.65 organism in 1 cc. Referring to the table, we see that for

$x = 0.0065$	$e^{-x} = 0.9935$
$x = 0.065$	$e^{-x} = 0.9371$
$x = 0.650$	$e^{-x} = 0.5222$
$x = 6.500$	$e^{-x} = 0.0015$

This means that for every 10,000 tubes inoculated with each of these dilutions, on the average, 15 of those inoculated with 10 cc., 5222 of those inoculated with 1.0 cc., 9371 of those inoculated with 0.1 cc., and 9935 of those inoculated with 0.01 cc. would show no growth; or if 100 tubes were inoculated with each dilution, one would expect that all the tubes receiving 10 cc., 48 receiving 1 cc., 6 receiving 0.1 cc., and 1 receiving 0.01 cc. would show growth. If a person wished to determine the most probable number of organisms by the result of inoculating a series of tubes with a single dilution, the best size of inoculum to use would in that case be 1 cc.

When a person inoculates a series of tubes with a single dilution and finds the percentage which show growth, he will also be able, by means of this table, to determine what size inoculum would have produced growth in 50 per cent of the tubes.

CASE III

In this case several dilutions are used, and several tubes are inoculated with each dilution.

For this development the following terms will be used:

w_1, $(1 - w_1)$ = the probability of a success and failure respectively, in a sample of size a_1,
w_2, $(1 - w_2)$ = the probability of a success and failure respectively, in a sample of size a_2,
w_3, $(1 - w_3)$ = the probability of a success and failure respectively, in a sample of size a_3,
n_1 = the number of samples of size a_1 that are taken,
n_2 = the number of samples of size a_2 that are taken,
n_3 = the number of samples of size a_3 that are taken,
p_1, q_1 = the number of failures and successes obtained out of n_1 trials.
p_2, q_2 = the number of failures and successes obtained out of n_2 trials,
p_3, q_3 = the number of failures and successes obtained out of n_3 trials.

The total probability will therefore be the product of several binomials, and by expanding each of them and multiplying the expanded forms together, the different terms in the product will be the probability of getting any set of combinations of failures and successes, as

$$p_1q_1;\ p_2q_2;\ p_3q_3;\ \ldots\ldots$$

The general formula for the probability of any one of these terms will then be:

$$P = \frac{(p_1+q_1)!}{p_1!\,q_1!}(w_1)^{q_1}(1-w_1)^{p_1}\frac{(p_2+q_2)!}{p_2!\,q_2!}(w_2)^{q_2}(1-w_2)^{p_2}\frac{(p_3+q_3)!}{p_3!\,q_3!}(w_3)^{q_3}(1-w_3)^{p_3}\ldots\ldots \quad (7)$$

This equation is obtained by multiplying together several equations, as (4), each being derived from a different binomial.

Now if the probabilities w_1, w_2, w_3, etc., are functions of x, it is possible to find the most probable values of x by differentiating the above equation with respect to x and putting the derivative equal to zero. This equation can be differentiated most readily by taking the logarithms of both sides, thus:

$$\ln P = \ln\frac{(p_1+q_1)!}{p_1!\,q_1!} + \ln\frac{(p_2+q_2)!}{p_2!\,q_2!} + \ln\frac{(p_3+q_3)!}{p_3!\,q_3!}\ldots\ldots + q_1\ln w_1 +$$

$$q_2\ln w_2 + q_3\ln w_3\ldots\ldots + p_1\ln(1-w_1) + p_2\ln(1-w_2) + p_3\ln(1-w_3)\ldots\ldots$$

Differentiating and substituting $n_1 - p_1$ for q_1, etc., we get

$$\frac{dP}{dx}\frac{1}{P} = d\frac{\ln w_1}{dx}\left[n_1 - \frac{p_1}{1-w_1}\right] + d\frac{\ln w_2}{dx}\left[n_2 - \frac{p_2}{1-w_2}\right] + d\frac{\ln w_3}{dx}\left[n_3 - \frac{p_3}{1-w_3}\right]\ldots\ldots$$

Now if x represents the percentage of a certain kind that are present in a unit, and a_1, a_2, a_3, etc., represent the number of these units that are selected, then

$$w_1 = (1-x)^{a_1};\ w_2 = (1-x)^{a_2};\ w_3 = (1-x)^{a_3};\ \text{etc.}$$

and

$$\frac{d\ \ln\ w_1}{dx} = \frac{-a_1}{1-x},\ \frac{d\ \ln\ w_2}{dx} = \frac{-a_2}{1-x},\ \frac{d\ \ln\ w_3}{dx} = \frac{-a_3}{1-x},\ \text{etc.}$$

Substituting these values in the above differential equation, putting it equal to zero, and factoring out all common multipliers, we get:

$$a_1\left(n_1 - \frac{p_1}{1 - w_1}\right) + a_2\left(n_2 - \frac{p_2}{1 - w_2}\right) + a_3\left(n_3 - \frac{p_3}{1 - w_3}\right) = 0$$

$$\frac{p_1 a_1}{1 - w_1} + \frac{p_2 a_2}{1 - w_2} + \frac{p_3 a_3}{1 - w_3} \ldots\ldots = a_1 n_1 + a_2 n_2 + a_3 n_3 \ldots\ldots \quad (8)$$

This is the same type of equation as that developed by Greenwood and Yule (1917), but is a little more general. With this formula a_1, a_2, a_3, etc., n_1, n_2, n_3, etc., are constants for any given experiment. p_1, p_2, p_3, etc., can be determined by experimental data. Since w_1, w_2, w_3, etc., are functions of x, it is then possible to solve for x the most probable value of that variable. The solution of the equation can be simplified if tables are worked out that give the relationship between x and w_1, w_2, w_3, etc.

To apply this to the problem of determining the most probable number of bacteria per cubic centimeter, let us assume that a set of tubes are inoculated with various amounts of the sample, one set inoculated with 10 cc. each, another set with 1 cc. each, and a third set with 0.1 cc. in each tube. In this case,

$w_1 = e^{-10x}$	$w_2 = e^{-x}$	$w_3 = e^{-\frac{x}{10}}$
$a_1 = 10$	$a_2 = 1$	$a_3 = 0.1$

Let us assume that ten tubes are used in each set, Then

$$n_1 = n_2 = n_3 = 10$$

The general formula then becomes

$$\frac{10\ p_1}{1 - e^{-10x}} + \frac{p_2}{1 - e^{-x}} + \frac{p_3}{10\left(1 - e^{-\frac{x}{10}}\right)} = 111$$

in which p_1 = the number of tubes receiving 10 cc. that show growth,

p_2 = the number of tubes receiving 1 cc. that show growth,

p_3 = the number of tubes receiving 0.1 cc. that show growth.

In the appendix, table 2, will be found values of $\frac{1}{1 - e^{-x}}$ for

the different values of x. With the aid of these tables it is an easy matter to solve the equation by trial and error by selecting the value of $\frac{1}{1-e^{-10x}}, \frac{1}{1-e^{-x}}, \frac{1}{1-e^{-\frac{x}{10}}}$, that will make the left hand side of the equation equal to 111. The values of x corresponding to these values of $\frac{1}{1-e^{-x}}$, etc., will be the most probable number of organisms per cubic centimeter.

To demonstrate the method of calculation, a specific example is included.

Suppose in an actual experiment 160 tubes were inoculated with each of a series of dilutions. Suppose that the following results were obtained:

DILUTION	NUMBER SHOWING GROWTH	NUMBER SHOWING NO GROWTH
10^4	160	0
10^5	160	0
10^6	158	2
10^7	61	99
10^8	8	152
10^9	0	160

The critical dilutions are therefore 10^6, 10^7, 10^8. The most probable number of organisms present in the 10^7 dilution can be obtained from the general equation:

$$\frac{a_1 p_1}{1-e^{-a_1 x}} + \frac{a_2 p_2}{1-e^{-a_2 x}} + \frac{a_3 p_3}{1-e^{-a_3 x}} = a_1 n_1 + a_2 n_2 + a_3 n_3$$

In this experiment, a_1 is 10, a_2 is 1, and a_3 is 0.1; $n_1 = n_2 = n_3 = 160$; $p_1 = 158$, $p_2 = 61$, $p_3 = 8$. Substituting these values in the above we get

$$\frac{10 \cdot 158}{1-e^{-10x}} + \frac{1 \cdot 61}{1-e^{-x}} + \frac{8}{10\left(1-e^{-\frac{x}{10}}\right)} = 160\ (10 + 1 + 0.1)$$

$$\frac{1580}{1-e^{-10x}} + \frac{61}{1-e^{-x}} + \frac{8}{10\left(1-e^{-\frac{x}{10}}\right)} = 1776.0$$

An approximate idea of the value of x can be obtained by considering the middle dilution in which the proportion of tubes

showing no growth is $\frac{99}{160} = 0.6188$. From table 1 (appendix) the value of $e^{-x} = 0.6188$ is found to correspond to a value of $x = 0.4800$. By obtaining the reciprocals of $1 - e^{-10x}$, $1 - e^{-x}$, and $1 - e^{-\frac{x}{10}}$ from table 2 (appendix) and substituting in the above equation, we can obtain a more accurate value of x. Substituting the proper reciprocals in the left hand side of the equation and solving, we get

1776.898 for $x = 0.4650$
1774.598 for $x = 0.4700$
1770.194 for $x = 0.4800$

By interpolation, we find that the function becomes

1776.00 for $x = 0.4669$

In the case of the ten tubes inoculated with each of the dilutions this equation has been solved for all the combinations that are likely to occur. Table 3 (appendix) shows the most probable number of organisms which correspond to each of the combinations.

When 10 tubes are used in each of these dilutions, and when $a_1 = 10$ cc., $a_2 = 1$ cc., and $a_3 = 0.1$ cc., the general equation (7) becomes

$$P = \frac{10!}{p_1!\,q_1!}\frac{10}{p_2!\,q_2!}\frac{10}{p_3!\,q_3!}\left(e^{-10x}\right)^{q_1}\left(e^{-x}\right)^{q_2}\left(e^{-\frac{x}{10}}\right)^{q_3}\left(1-e^{-10x}\right)^{p_1}\cdot\left(1-e^{-x}\right)^{p_2}\cdot\left(1-e^{-\frac{x}{10}}\right)^{p_3}$$

Taking the log to the base 10 of both sides of the equation, we get

$$\log P = \log\frac{10!}{p_1!\,q_1!} + \log\frac{10!}{p_2!\,q_2!} + \log\frac{10!}{p_3!\,q_3!} - x\left(10q_1 + q_2 + \frac{q_3}{10}\right)\log e +$$

$$10p_1\log\left(1-e^{-10x}\right) + p_2\log\left(1-e^{-x}\right) + p_3\log\left(1-e^{-\frac{x}{10}}\right)$$

By means of this equation it is possible to solve for the value of P for any given value of x and p_1, p_2, and p_3. This has been done for all the combinations where P has a value greater than 0.01 per cent. These values show how often a certain combination can be expected if the number of organisms in the solution are as indicated by the most probable values of x. These frequencies

are helpful in interpreting data. If certain combinations are found to occur more often than indicated by these values of P, one may become suspicious that something is wrong either with the technic or with the medium.

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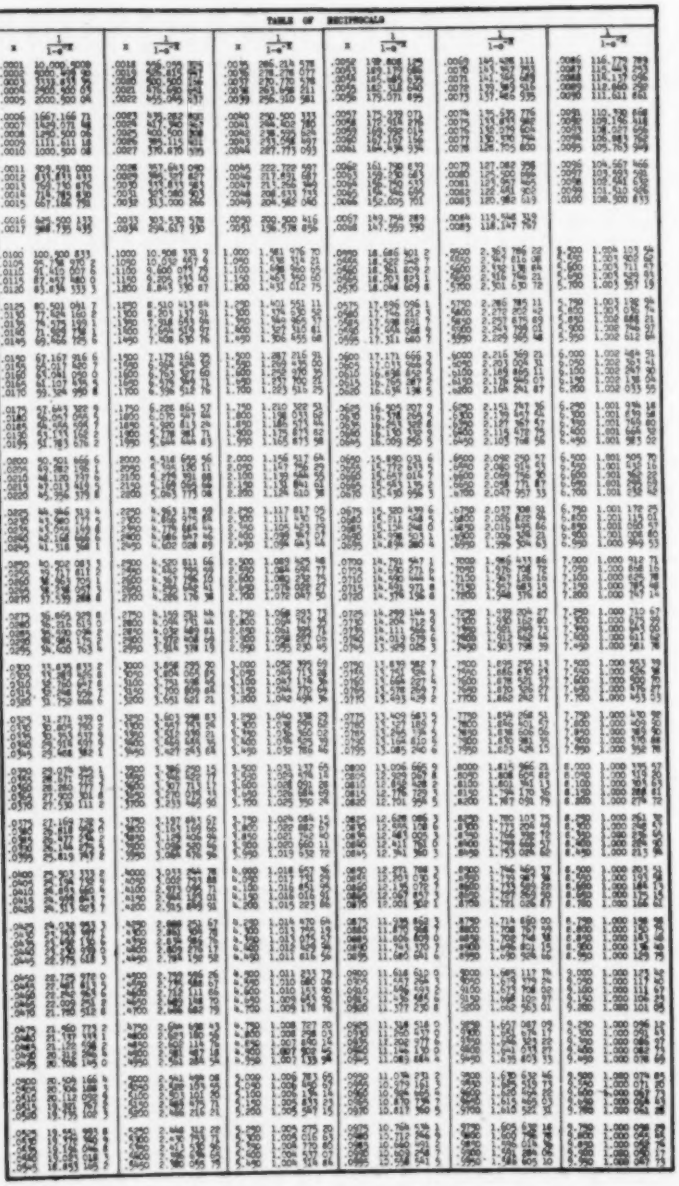
TABLE OF EXPONENTIAL FUNCTIONS



PROBABILITY TABLE A

Code	X	P	Code	X	P	Code	X	
10 10 10	100.0	100.0	10 10 0	2.40	3.4	10 9 0	1.70	5.30
10 10 9	23.0	96.7	10 9 9	9.07	0.01	10 8 7	1.10	0.01
10 10 8	19.2	95.2	10 9 8	5.63	0.04	10 8 8	2.78	0.14
10 10 7	12.0	95.2	10 9 7	3.93	0.27	10 8 7	2.49	0.56
10 10 6	9.18	93.0	10 9 6	3.08	0.85	10 8 6	2.21	1.54
10 10 5	7.02	94.2	10 9 5	2.46	2.25	10 8 5	1.96	4.50
10 10 4	5.42	93.4	10 9 4	2.08	4.85	10 8 4	1.71	8.45
10 10 3	4.38	94.7	10 9 3	2.04	8.57	10 8 3	1.50	19.81
10 10 2	3.49	17.1	10 9 2	2.28	11.77	10 8 2	1.30	7.21
10 10 1	2.75	10.9	10 9 1	1.97	11.02	10 7 6	2.19	0.01
10 7 5	1.26	0.20	10 6 3	1.25	0.59	10 4 5	1.073	0.01
10 7 4	1.21	0.80	10 7 2	1.05	2.15	10 4 4	0.845	0.07
10 7 3	1.13	2.57	10 7 1	0.913	8.45	10 4 3	0.618	0.43
10 7 2	1.15	2.17	10 6 0	0.768	10.73	10 4 2	0.700	2.26
10 7 1	1.15	10.01	10 5 5	1.30	0.02	10 4 1	0.589	7.97
10 7 0	1.01	9.13	10 5 4	1.16	0.14	10 4 0	0.493	14.59
10 6 6	1.75	0.01	10 5 3	1.02	0.91	10 3 3	0.773	0.06
10 6 5	1.47	0.08	10 5 2	0.872	3.30	10 3 2	0.660	0.46
10 6 4	1.21	0.11	10 5 1	0.745	8.94	10 3 1	0.561	1.80
10 6 3	1.25	0.59	10 5 0	0.622	12.66	10 3 1	0.474	6.93
10 3 0	.332	17.67	10 0 2	.114	0.15	9 5 1	.372	0.85
10 2 3	.542	0.01	10 0 1	.098	1.42	9 5 0	.305	1.78
10 2 2	.456	0.12	10 0 0	.091	9.35	9 4 2	.266	0.02
10 2 1	.388	5.30	9 8 1	.488	0.02	9 4 1	.326	1.13
10 2 0	.302	18.14	9 7 1	.488	0.02	9 4 0	.260	3.56
10 1 2	.456	0.04	9 7 0	.430	0.17	9 3 2	.220	0.04
10 1 1	.376	0.80	9 6 2	.430	0.56	9 3 1	.264	0.26
10 1 0	.276	15.14	9 6 1	.375	0.23	9 3 0	.205	6.23
			9 6 0	.314	0.56	9 2 1	.208	1.77
			9 5 2	.316	0.10	9 2 0	.155	6.23
9 2 2	.316	0.03	8 6 0	.270	0.11	8 2 2	.210	0.14
9 2 1	.262	0.20	8 5 1	.267	0.11	8 2 1	.168	1.24
9 2 0	.205	2.19	8 5 0	.205	0.42	8 2 0	.155	7.12
9 1 2	.264	0.53	8 4 2	.266	0.04	8 1 2	.167	0.11
9 1 1	.205	0.19	8 4 1	.205	0.33	8 1 1	.126	1.45
9 1 0	.151	1.84	8 4 0	.217	1.67	8 1 0	.117	9.14
9 0 1	.151	0.03	8 3 1	.179	0.08	8 0 0	.119	0.06
9 0 2	.217	0.07	8 3 2	.214	0.77	8 0 1	.145	0.88
9 0 1	.151	0.79	8 3 0	.143	3.73	8 0 0	.126	6.41
9 0 0	.154	5.02	8 2 3	.255	0.01	7 6 0	.212	0.02
7 5 1	.209	0.08	7 2 0	.133	5.49	6 4 0	.170	0.32
7 5 0	.161	0.16	7 1 2	.145	0.26	6 3 2	.153	0.01
7 4 2	.208	0.01	7 1 1	.138	1.10	6 3 1	.136	0.20
7 4 1	.168	0.12	7 1 0	.116	8.36	6 3 0	.123	1.35
7 4 0	.171	0.66	7 0 2	.138	0.04	6 2 2	.117	0.01
7 3 2	.168	0.03	7 0 1	.116	0.80	6 2 1	.102	0.04
7 3 1	.159	0.35	7 0 0	.101	7.92	6 2 0	.107	4.12
7 3 0	.155	2.44	6 5 0	.155	0.06	6 1 2	.121	0.06
7 2 2	.157	0.04	6 4 2	.171	0.00	6 1 1	.105	0.87
7 2 1	.150	0.78	6 4 1	.155	0.04	6 1 0	.092	8.75
6 0 2	.105	0.04	5 2 0	.086	3.36	4 3 0	.080	0.43
6 0 1	.092	0.83	5 1 2	.099	0.53	4 2 2	.080	0.16
6 0 0	.078	9.77	5 1 1	.086	0.58	4 2 1	.068	2.16
5 5 0	.126	0.04	5 1 0	.079	8.28	4 2 0	.080	0.01
5 4 2	.127	0.02	5 0 2	.086	0.01	4 1 2	.068	0.50
5 4 1	.114	0.16	5 0 1	.072	0.73	4 1 1	.058	7.21
5 4 0	.113	0.08	5 0 0	.069	12.10	4 0 2	.067	0.02
5 3 2	.100	0.79	4 3 0	.105	0.02	4 0 1	.056	0.76
5 3 1	.113	0.01	4 2 2	.093	0.06	4 0 0	.045	14.52
5 2 2	.099	0.28	4 2 1	.080	0.03	3 4 0	.076	0.04
3 3 2	.086	0.00	3 0 0	.032	18.64	1 2 1	.038	0.02
3 3 1	.075	0.02	2 4 0	.062	0.02	1 2 0	.029	0.53
3 3 0	.074	0.23	2 3 0	.051	0.11	1 1 1	.028	0.12
3 2 1	.064	0.06	2 2 1	.051	1.96	1 1 0	.019	4.48
3 2 0	.053	1.53	2 2 0	.045	0.62	1 0 0	.019	0.48
3 1 2	.064	0.01	2 1 0	.030	6.01	1 0 0	.115	34.58
3 1 1	.053	0.31	2 0 2	.045	0.21	0 2 0	.018	0.21
3 1 0	.043	2.50	2 0 1	.030	0.41	0 1 1	.018	0.04
3 0 2	.053	0.09	2 0 0	.020	24.11	0 1 0	.009	0.92
3 0 1	.042	0.51	1 3 0	.038	0.04	0 0 1	.009	0.26

This table indicates the number of bacteria per cubic centimeter based on the number of tubes showing growth which have been inoculated with dilutions as outlined. Ten tubes are to be inoculated with 10 cc. each, ten tubes with 1 cc. each, and ten tubes with 0.1 cc. each. The code number is made up of the number of tubes showing growth in each case, the first number of the code representing the number of tubes showing growth that are inoculated with 10 cc. each, the second those inoculated with 1 cc. each, and the last those inoculated with 0.1 cc. each. The column labeled *X* then gives the most probable number of bacteria per cubic centimeter in the material used for inoculation. The column labeled *P* gives the percentage of times that the code would be obtained if an infinite number of determinations were made of a solution containing the number of organisms indicated by *X*.

Example: Suppose that a culture is inoculated into broth in a series of 10 dilutions in steps of 10. Assume that 9 of the tubes inoculated with 10^{-7} cc., that 3 of the tubes inoculated with 10^{-8} cc. and that none of the tubes inoculated with 10^{-9} cc. show growth. The code will then be 9 3 0. Referring to the table, *X* is found to be 0.255. This means that the most probable number of bacteria in the 10^{-8} cc. dilution was 0.255 bacteria per cubic centimeter, or that the most probable number in the original solution was 0.255 times 10^{8} or 25,500,000. If this experiment were repeated an infinite number of times on a solution containing this number, this result would be obtained 6.23 per cent of the time.

If 5 tubes are used in each dilution instead of 10, then multiply the code obtained by two, and refer to the table, as above. In this case, however, the column labeled *P* does not apply.

RELATION OF CHANGES IN MORPHOLOGY AND METABOLISM IN B. COLI

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Received for publication April 2, 1932

In order to gain some knowledge concerning factors which might influence morphological variations of microörganisms a series of experiments were undertaken in which the time of growth, the nature of the media, and metabolism (together with the products formed during growth) were studied.

Preliminary study was made using the usual laboratory media; however it was evidently necessary to cultivate the organism in a synthetic medium in which the metabolism as well as the morphological changes could be more easily followed. While trying to develop such media it was found that Koser's Citrate Media (1923) provided an excellent means for following morphological changes in both fecal and non-fecal *B. coli*. It was also found that a medium containing both dextrin and tartrate was a satisfactory one, in which *B. coli* and *B. alcaligenes* grew actively and in which changes in morphology and metabolism could be traced.

The changes in morphology of the organism were studied by staining by Gram's method. This method was adopted because it proved amply sufficient to show the alterations in morphology and also showed changes in gram responses which seemed to accompany morphological variations within the organism.

As a preliminary procedure in order to study the morphological changes, cultures of *Bacillus coli*, *Bacillus typhosus*, and *Bacillus alcaligenes*, each of which had been developed from a single cell according to the modification of the Orskow Single Cell Technique by Stearns and Stearns (1927), were grown in nutrient broth and nutrient agar with and without the addition of glu-

cose (prepared according to standard methods). These cultures were examined at the end of twenty-four hours and then at forty-eight-hour intervals thereafter for a period of eighteen days.

Buchanan's method was the one used as a routine in the Gram stain. This method was checked at regular intervals by Hucker's method using acetone as a decolorizing agent and also by the method of Kemp & Fleisher in which anilin is used as the decolorizing agent. In case of doubt all three methods were used and no results were accepted unless there was complete agreement.

The organisms grown in the same media and of the same age were all stained on a slide marked into divisions, together with controls (twenty-hour agar cultures of *Staphylococcus aureus* and *Bacillus coli*). The same procedure was carried out with organisms grown in the various media. No results were accepted unless the control smears of twenty-hour agar cultures showed typical gram reactions.

All of the Gram-negative organisms show as they become older variations in morphology such as greatly enlarged, lanceolate, filamentous, and drumstick-like forms; also they stain less intensely as was shown by Henerici (1926). In addition granular, lanceolate, and drumstick-like forms containing within the cell, masses of Gram-positive material were observed. These usually appeared in successive order and then disappeared, leaving the Gram preparation almost void of material with the exception of fine granular well distributed masses resembling blue pin point dots. These pin point dots might be regarded as débris within the culture but when such cultures were transplanted to agar a profuse growth was obtained. The abnormal forms also gave a profuse growth when transplanted to agar but at times the growth in the transplanted culture was much slower than when transplantation from the cultures showing the pin point dot was made.

The influence of the various media on morphology and gram reaction was evident in the fact that Gram-negative organisms retained the normal characteristics longer when grown on solid media than when grown in liquid media. Also in media enriched with glucose the organisms retained their normal characteristics longer than in media practically free from that sugar.

These changes in morphology and gram responses having been observed when the organisms were grown in the ordinary laboratory media, efforts were made to determine if possible the relationship of certain factors to such changes. For this purpose

TABLE 1

Showing various combinations of synthetic media

Ingredients are shown in grams per 1000 cc.

no.	Su.	Dr.	Gl.	Gly.	KH_2PO_4	K_2HPO_4	$MgSO_4$	NH_4Cl	$CaCl_2$	$H(NH_4)_2PO_4$	NaCl	$NH_4Tar.$
1	10	10	10	10	0.5	0.5	0.1	1.5				
2	10	10	10		0.5	0.5	0.1	1.5				
3	10	10			0.5	0.5	0.1	1.5				
4	10				0.5	0.5	0.1	1.5				
5		10	10	10	0.5	0.5	0.1	1.5				
6			10	10	0.5	0.5	0.1	1.5				
7				10	0.5	0.5	0.1	1.5				
8		10	10		0.5	0.5	0.1	1.5				
9			10		0.5	0.5	0.1	1.5				
10		10			0.5	0.5	0.1	1.5				
11	10				0.5	0.5	0.1	1.5				
12	10	10	10	10	0.25	1.25	0.1	0.75				
13	10	6				1.0		2.0	0.1		0.02	
14	10		6			1.0		2.0	0.1		0.02	
15	10					1.0		2.0	0.1		0.02	
16				10		1.0				1.0	0.02	
17	5			5		1.0				1.0	0.02	
18	10					1.0	0.1		0.1			2.0
19						1.0	0.1		0.1			5.0
20			10			1.0	0.1		0.1			10.0
21				10		1.0	0.1		0.1			10.0
22		10			1.0	1.0	0.1		0.1			10.0
23					1.0	1.0	0.1		0.1			10.0
24					1.0	1.0	0.1		0.1			10.0

Note: No., number of media; Su., sucrose; Dr. dextrin; Gl., glucose; Gly., glycerol.

the ordinary laboratory media were not satisfactory and it was necessary to develop a simple synthetic media which would serve for the purpose of tracing morphological changes in relation to metabolism.

Media composed of numerous combinations of simple substances were tried as is shown in table 1. None but C.P. chemicals previously assayed were used. Some of the assays showed traces of iron and sodium chloride but neither of these were considered objectionable when present in small quantities. All media were sterilized at 10 pounds pressure for fifteen minutes. Higher pressure seemed to cause excessive hydrolysis of the carbohydrates.

To test the ability of the media to maintain growth, all of the organisms were carried through three successive twenty-four-hour transplants in each medium and were stained at the end of each twenty-four hours. In the majority of cases the organisms

TABLE 2

Growth and Gram reaction of B. coli, B. alcaligenes, and B. typhosum in a third transplant of the various media under investigation

	NUMBER OF MEDIA																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
B. coli....	−	−	−	−	−	0	−	+	+	−	Po.	Po.	−	−	−	0	0	−	0	−	−	−	0	0
B. ty.....	0	0	0	0	0	0	0	0	0	0	0	0	Br.	+−	0	0	0	0	0	0	0	0	0	0
B. alc.....	0	0	−	+	−	0	+	−	−	0	+−	0	−	−	0	0	0	0	0	0	0	−	0	0

0, no growth; +, gram-positive, −, Gram-negative; Br. organism stained brown, Po., increased in size, drumstick like formation with centers of enlarged ends staining Gram-positive.

showed multiplication in the first transplant but in many cases the gram reaction was altered. In a few exceptional cases growth was observed in the second transplant when the organism did not grow in the third transplant.

The growth and Gram reactions of the various organisms in the third transplant are shown in table 2 which is self-explanatory as to which media will support the growth of the organisms under investigation. It may be stated that not all organisms grow equally well in any one of the media nor does any organism grow in all the combinations. Each organism has a medium in which it thrives best. The group of Gram-negative organisms tested seem to have a preference for medium 22.

The fact that these organisms continue to grow in the media is indicative that they must utilize the ammonia radicle of either ammonium chloride or ammonium tartrate as a source of nitrogen since it is the only source for that element.

While trying to develop a suitable synthetic media it was observed that all organisms and especially *B. coli* showed the greatest amount of morphological variation in those media which did not actively support growth of the organisms. To verify the above suggestion attention was directed to Koser's Citrate Medium as it seemed to provide an excellent means for the study and comparison of two closely related strains of *B. coli*, the one a fecal the other a non-fecal strain, with reference to changes which might occur within the cell when an organism is grown under favorable and unfavorable conditions.

Seven strains of *B. coli* which had been secured from various sources were investigated. Six of the strains proved to be fecal types while the seventh was non-fecal. The six strains of fecal *B. coli* showed no growth in Koser's Citrate Medium over a period of eleven days; even when the medium was heavily inoculated to a stage of turbidity the tubes cleared within a few days. When these eleven-day cultures of fecal types were Gram-stained nothing could be seen on the slide except what appeared to be tiny blue Gram-positive dots. However, when transplants were made from these cultures to agar slants an abundant growth was obtained within twenty-four hours even from the eleventh daily transfer.

From the above observations it appears that fecal strains of *B. coli* must undergo some changes after being inoculated into Koser's Citrate Medium. To determine how rapidly such changes occurred it seemed advisable to prepare new cultures and examine them at two-hour intervals. The results are shown in table 3 together with that of the non-fecal strain which was treated in a like manner.

Whenever the organisms were examined as reported in table 3 transplants were made to fresh agar slants to determine if these forms would develop into typical *B. coli*. The growth was slow during the first hours after transplanting but the cultural seemed

to regain what it had lost during the latter part of the twenty-four hour period.

Non-fecal strains of *B. coli* were now grown in Koser's Citrate Medium and examined daily. The hydrogen ion concentration of the media was also determined. The results are shown in table 4.

Table 4 shows that while in general the pH rises as the organisms continue to grow in the media, there are however alternate days during which the pH does not change. One might assume that the organisms were not actively growing during this time.

TABLE 3

Showing the action of fecal and non-fecal B. coli when grown in Koser's Citrate Media and examined at intervals of two hours

	TIME	GRAM	CHARACTER OF ORGANISMS
	hours		
Fecal *B. coli*	2	Positive	Perfect rods, granular
	4	Positive	Distorted rods which had stained various shades of blue. Some large blue spherical forms
	6		Small blue pin point dots
	8		Small blue pin point dots
Non-fecal *B. coli*	2	Negative	Normal rods, small
	4	Negative	Normal rods, small
	6	Negative	Normal rods, normal size
	8	Negative	Normal rods, normal size

At the beginning of each one of these periods during which the hydrogen ion concentration showed little or no change, the Gram preparations showed greatly distorted rods with enlarged ends containing masses of Gram-positive material while at the beginning of the next rise in pH the organisms appeared to be almost typical normal rods. This alteration in form occurred only in the earlier period that is through the eighth day in the particular experiment cited.

The observations concerning *B. coli* grown in Koser's Citrate media, can be stated in the following summary.

Fecal *B. coli* when grown in Koser's Citrate Media alter their Gram reaction within two hours and then change their morphology to the extent that no normal or abnormal rods may be detected; only blue-staining gram-positive small granules can be demonstrated.

TABLE 4

Showing variations of non-fecal B. coli when grown for a number of days in Koser's Citrate Media

DAYS	PH.	GRAM	DESCRIPTION OF ORGANISM
0	6.8	−	Normal colon rods
1	7.2	−	Normal colon rods, somewhat granular
2	7.7	−+	Greatly distorted with enlarged ends staining Gram-positive
3	7.7	−	Normal colon rods
4	7.9	−+	Distorted forms with enlarged ends containing Gram-positive material
5	7.9	−	Mostly normal colon rods
6	8.4	−	Granular distorted forms
7	8.4	−	Granular and normal forms
8	8.4	−	Normal forms
9	8.4	−	Granular enlarged forms
10	8.7	−	Granular enlarged forms
11	8.9	−	Granular enlarged forms
12	8.9	−	Granular enlarged forms
13	8.9	−+	Granules have collected at ends and stain Gram-negative
14	8.9	−+	Granules have collected at ends and stain Gram-positive. The remainder of the cell stains Gram-negative
15	8.9	−+	Many distorted forms with enlarged ends, others are coccoid, a few Gram-negative rods

When such a culture of fecal *B. coli* in Koser's Citrate media is transplanted to a fresh agar slant an abundant growth is produced. This growth is much heavier than might normally be expected.

Non-fecal *B. coli* in Koser's Citrate Media continue to grow over a period of days but show alternation of normal and abnormal forms and this alternation seems to be accompanied by changes in the hydrogen ion concentration of the media. The

periods are shorter and more distinct during the first six days of growth than after that time.

The above observations on non-fecal *B. coli* grown in Koser's Citrate Media seemed to link changes in the hydrogen ion concentration of the media with morphological changes of the organism but whether the morphological changes had been influenced by the metabolism was not here determined. Thus, further experimentation was carried out with medium 22, firstly because it actively supported the growth of *B. coli* and *B. alcaligenes*; second, because it contained a dual source of carbon (dextrin and tartrate ion) which if alternately metabolized might influence the hydrogen ion concentration of the media; thirdly, because it consisted of simple chemical compounds whose disappearance from the media could readily be followed.

The seven strains of *B. coli* and *B. alcaligenes* were grown in medium 22 which consisted of the following ingredients.

Dextrin	10.0 grams
Dibasic potassium phosphate	1.0 gram
Monobasic potassium phosphate	1.0 gram
Magnesium sulphate	0.1 gram
Calcium chloride	0.10 gram
Ammonium tartrate	10.0 grams
Distilled water	1,000.0 cc.

The organisms were grown in 500-cc. Erlenmeyer flasks containing 400 cc. of the media. Daily, sufficient media was removed with a sterile pipette for the following tests.

pH—hydroquinone potentiometer.
Gram reaction—Buchanan's method verified by the methods of Hucker and Kemp and Fleisher.
Morphology—observations under microscope using a 90× appochromat objective and a 20× ocular.
Dextrin—spot plate using a weak Lugol's solution.
Reducing sugars—Benedict's test.
Lactic acid—Uffleman's reaction. Spot plate.
Ammonia—Nessler's reaction. Spot plate.
Nitrates—diphenamine reagent and two drops of sulphuric acid.
Nitrites—solution I, 0.5 per cent sulphanilic acid in 5 N acetic acid. Solution II, dimethyl-alpha-naphthyl-amine 6 cc. in 1 liter of 5 N acetic acid. The medium was put on a spot plate and the solutions added in order.

Amino acids—ninhydrin test. The organisms were removed by the aid of a Seitz filter before these tests were made.

Tartrates—formation of copper complexion. This test is not specific since the copper ion complex is formed by any alpha-beta-hydroxy-acid, but negative tests may be accepted as denoting the absence of the tartrate ion.

Purity of culture—usual methods.

Phage—observation of moth eaten patches on agar slants and also by adding the filtrate to actively growing broth cultures.

The results may in general be considered as typical reactions of *B. coli* in the media as demonstrated in repeated experiments. The same strain in the same medium did not show in each test absolutely identical results; such minor irregularities could be expected since in an experiment of this nature it is impossible to control accurately all factors involved; yet, the results were similar and no evidence was found in one determination that could not be observed in the others. The different strains showed no variations other than that within the strain and here as before the results were consistent. The results obtained when *B. coli* was grown in media 22 are shown in table 5.

The six other strains of *B. coli* and *B. alcaligenes* gave similar results, with the exception that in a few cases nitrites appeared on the second day and disappeared on the sixth day.

The daily variation of pH in the media is best demonstrated by a curve in which the pH is plotted against time. The curve shows an initial fall of pH and then alternating rises and falls in its value. Figure 1 shows the daily variations when *B. coli* was grown in medium 22 having an initial pH of 6.8. In the majority of cases the general trend of the curve was towards the alkaline limits of growth; however, in a few cases the curve finally terminated in the acid range. The cause for *B. coli* terminating its activity when grown in the same medium, on one occasion in the alkaline range and at another time in the acid range was not determined, however, sufficient data were gathered to state that apparently it was not due to slight variations in the initial pH nor to the initial fall in its value. It appears as though it might be a factor of metabolism but the analyses of the media show no evidence in this direction.

Table 5 shows the simultaneous disappearance of dextrin and

TABLE 5

Showing the action of B. coli in medium 22

DAYS	PH.	Gr.	DESCRIPTION OF ORGANISM	Ta.	Pu.	Ph.
0	6.78					
1	5.54	−−	Polar, granular			
2	6.15	+	Cocci, P in N, small blue dots			
3	5.51	−+	Distorted, some blue, many normal			
4	7.21	−+	Granular, P in N, many normal			
5	8.00	−+	Normal, somewhat enlarged	+	+	−
6	8.00	−	Normal forms			
7	7.34	−	Granular, polar			
8	5.64	−−	Normal			
9	6.92	−+	Distorted, normal, P in N	+	+	−
10	*8.07	−−	Normal			
11	*9.20	−−	Normal			
12	*8.07	−−	Normal			
13	*8.07	−+	Distorted, some normal	+	+	−
14	*9.20	−−	Normal			
Con.	6.75					

Analysis of media

DAYS	Dext.	RS.	La.	NH_4	NO_2	NO_3	AA.	Tart.
0	++++	−	−	++	−	−	−	++++
1	++++	−	++	++	−	−		++++
2	++++	−	++	++	−	−		++++
3	++++	−	+	++	−	−		++++
4	++++	−	+	++	−	−		++++
5	++++	−	+	++	−	−		+++
6	++++	−	+	++	−	−		+++
7	+++	−	+	++	−	−		++
8	+	−	−	+	−	−		++
9	−	−	−	+	−	−		+
10	−	−	−	+	−	−		−
11	−	−	−	+	−	−		−
12	−	−	−	+	−	−		−
13	−	−	−	+	−	−		−
14	−	−	−	−	−	−	−	−
Con.	++++	−	−	++	−	−		++++

Days, days after inoculation; PH, hydrogen ion concentration; Gr., reaction to Gram staining; Ta, result of transplantation to agar media; Pu., purity of culture; Ph., presence of bacteriophage;

Dext., dextrin; RS., reducing sugars; LA., lactic acid; NH_3, ammonia; NO_2, nitrites; NO_3, nitrates; AA., amino acids; Tart., tartrates; Con., control.

+, positive; −, negative; −− both Gram-positive and Gram-negative, * before a PH value indicates that the media oxidized the hydroquinone and that the results are only approximate.

the tartrate ion from the media; consequently the alternate metabolism of these two substances cannot be responsible for the variations in the pH curve and the morphological changes which accompany it.

When the initial pH of the media was increased to 8.2 by the addition of sodium hydroxide, many of the cultures gave Gram-positive reactions at the end of twenty-four hours especially in

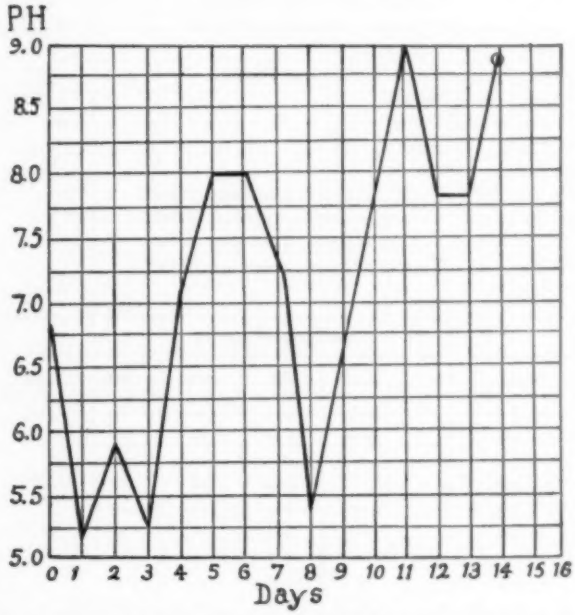

FIG. 1. SHOWING PH PLOTTED AGAINST TIME WHEN B. COLI IS GROWN IN MEDIUM 22

those cases where the growth of the organism was insufficient to cause a lowering of pH much below that of neutrality. These observations seem to support the evidence presented by Stearns and Stearns (1924) that organisms alter their Gram reaction as they cross their isoelectric point; however in this case the organisms remained viable as was shown by their continued growth in the media and also by transplants to agar slants.

TABLE 6

Showing the action of B. coli when grown in medium 22

DAYS	PH.	Gr.	DESCRIPTION OF ORGANISM	TA.	PU.	Ph.
0	8.20					
1	6.53	++	Normal, some coccoid	+	+	−
2	6.59	+	Blue dots, an occasional cell			
3	6.71	−	Small normal, small cocci, few distorted			
4	6.50	−+	Normal, distorted, coccoid			
5	6.50	−−	Enlarged, granular uniform	+	+	−
6	6.34	−−	Normal forms			
7	*8.66	−−	Small normal but thicker			
8	*8.84	−−	Normal			
9	*7.00	−−	Normal	+	+	−
10	*7.60	−−	Normal			
11	*8.50	−−	Normal			
12	*8.32	−+	Weakly stained, granular, polar	+	+	−
13	*8.70	−+	Weakly stained, granular, polar			
14	*8.95	−+	Weakly stained, granular, polar			
15	*8.66	−+	Weakly stained, granular, polar			
Con.	8.20					

Analysis of media

DAYS	Dext.	RS.	LA.	NH_3	NO_2	NO_3	AA.	Tart.
0	++++	−	−	++	−	−		++++
1	++++	−	++	++	−	−		++++
2	++++	−	+	++	−	−		++++
3	++++	−	−	++	−	−		++++
4	++++	−	−	++	−	−		++++
5	+++	−	−	++	−	−		+++
6	+++	−	−	++	−	−		+++
7	+++	−	−	++	−	−		+++
8	−	−	−	++	−	−		+++
9	−	−	−	++	−	−		+
10	−	−	−	++	−	−		+
11	−	−	−	++	−	−		+
12	−	−	−	++	−	−	−	+
13	−	−	−	++	−	−		+
14	−	−	−	++	−	−		+
15	−	−	−	+	−	−		−
Con.	++++	−	−	++	−	−		++++

The results shown in this series of experiments are very similar to those shown in table 5 with the exception that in this case the

pH curves ended in the alkaline range whereas some with an initial pH below seven ended in the acid range. Lactic acid appears in the media during the early period of growth indicating that its formation gives rise to the initial acidity. Tests show its later disappearance from the media which on the other hand suggests that it is an intermediate product in the metabolism of dextrin by *B. coli*.

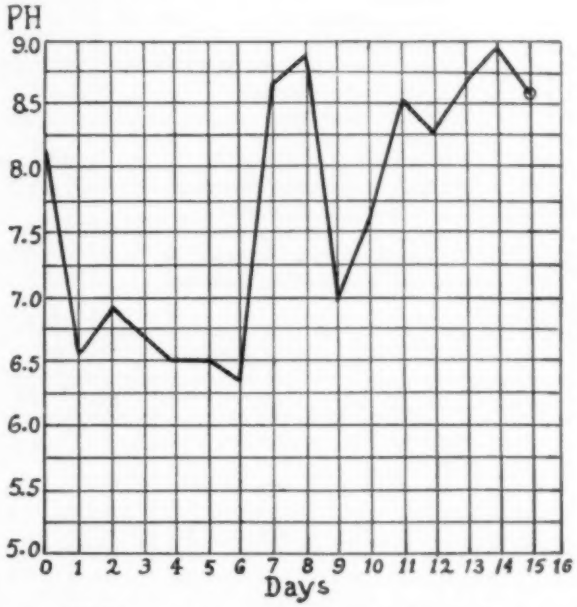

FIG. 2. SHOWING PH PLOTTED AGAINST TIME WHEN B. COLI IS GROWN IN MEDIUM 22 (pH B. 2)

Glucose, usually considered as an intermediate product of metabolism especially in cases where carbohydrates like dextrin are utilized, was not found in the media during the two previous experiments. If dextrin is not converted into glucose before it is metabolized by *B. coli*, this would appear to be a fact contrary to our knowledge of carbohydrate metabolism and yet the analysis of the media failed to show the presence of glucose. Thus, to determine if glucose was metabolized a series of determinations

were carried out in which glucose was substituted for dextrin in medium 22.

TABLE 7

Showing the action of B. coli in media 22 when glucose is substituted for dextrin

DAYS	PH.	Gr.	DESCRIPTION OF ORGANISM	AT.	PU.	PH.
0	6.80					
1	5.15	−−	Granular			
2	5.30	−−	Polar, granular			
3	5.10	−	Polar, granular			
4	5.30	−+	Polar, granular, large coccoid forms	+	+	−
5	5.30	−−	Mostly normal forms			
6	5.15	−−	Normal but larger			
7	5.40	−−	Mostly normal forms			
8	4.90	−+	Distorted, granular, polar	+	+	−
9	5.20	−	Enlarged, granular, distorted			
10	5.40	−	Distorted, poorly stained			
11	5.25	−+	Distorted, blue dots, polar			
12	5.20		Nothing on slide but blue dots	+	+	−
13	4.80	−+	Some normal, others distorted			
Con.	6.79					

Analysis of media

DAYS	Dext.	RS.	LA.	NH_4	NO_2	NO_3	AA.	Tart.
0	−	−+++	−	++	−	−		++++
1	−	++++	+	++	−	−		++++
2	−	++++	++	++	+	−		++++
3	−	++++	++	++	+	−		+++
4	−	++++	++	++	+	−		+++
5	−	++++	+	+	−	−		++
6	−	++++	+	+	−	−		++
7	−	++++	+	+	−	−		++
8	−	++++	−	+	−	−		+
9	−	++++	−	+	−	−		+
10	−	++++	−	−	−	−		+
11	−	++++	−	−	−	−		−
12	−	++++	−	−	−	−		−
13	−	++++	−	−	−	−	−	−
Con.	−	++++	−	++	−	−		++++

The results are shown in table 7.

The organisms do not thrive as actively in medium 22 in which glucose is substituted for dextrin; however, the daily variations

in morphology, Gram reactions, and pH occur as in the proceeding experiments.

Table 7 apparently indicates that glucose is not utilized by *B. coli* when in combinations such as those represented in medium 22; the organisms must utilize the tartrate ion which fact is verified by its disappearance from the media. The sole utilization of the tartrate may account for the fact that the organisms

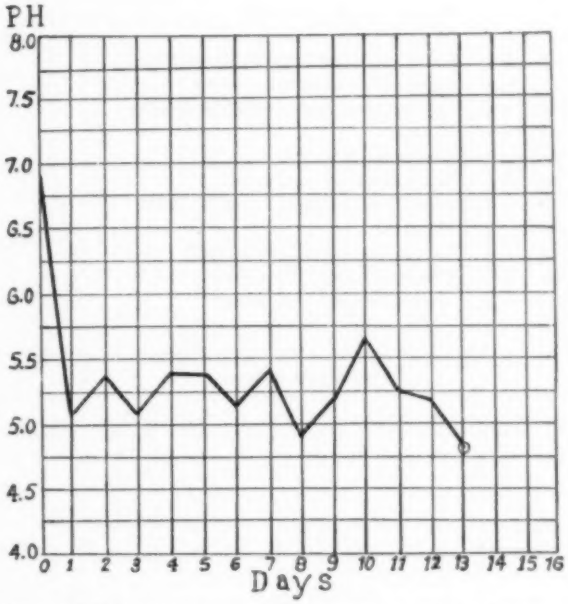

FIG. 3. SHOWING PH PLOTTED AGAINST TIME WHEN B. COLI IS GROWN IN MEDIUM 22 IN WHICH GLUCOSE HAS BEEN SUBSTITUTED FOR DEXTRIN

thrive less actively and naturally smaller variations would be expected in the pH curve. This was found to be true as may be seen from the curves shown in figure 3.

Having found that dextrin and the tartrate ion are both metabolized by *B. coli* but that glucose was not necessarily an intermediate product, further experimentation was carried out to determine if the tartrate ion had a definite influence on this

reaction. Thus ammonium chloride was substituted for ammonium tartrate in medium 22. The results are shown in table 8.

TABLE 8

Showing the action of B. coli in medium 22 when ammonium chloride is substituted for ammonium tartrate

DAYS	PH.	Gr.	DESCRIPTION OF ORGANISM	AT.	PU.	PH.
0	6.80					
1	5.35	−+	Heavily polar, coccoid			
2	5.35	−+	Heavily polar, coccoid	+	+	−
3	5.85	−+	Large globular forms, coccoid			
4	5.25	−+	Normal, some coccoid forms			
5	5.00	−+	Few normal, many small blue dots			
6	5.00	−−	Normal			
7	5.25	−+	Distorted, coccoid, P in N			
8	5.30	−−	Normal, mostly enlarged			
9	5.55	−+	Blue dots, not much seen in field			
10	5.00	−−	Normal			
11	5.70	−+	Large blue cocci, few in field	+	+	−
12	5.05	−+	Distorted, small blue dots			
13	5.05	−+	Normal some cocci, polar	+	+	−
Con.	6.75					

Analysis of media

DAYS	Dext.	RS.	LA.	NH_3	NO_2	NO_3	AA.	Tart.
0	++	−	−	−	−	−	−	−
1	++	−	−	+	−	−		−
2	++	−	−	+	+	+		−
3	++	−	−	+	+	−		−
4	++	−	−	+	−	−		−
5	++	−	−	+	−	−		−
6	++	−	−	+	−	−		−
7	++	−	−	+	−	−		+
8	++	−	−	+	−	−		+
9	++	−	−	+	−	−		+
10	++	−	−	+	−	−		+
11	+	−	−	+	−	−		+
12	+	−	−	+	−	−		−
13	+	−	−	−	−	−		−
Con.	++	−	−	+	−	−		−

In this series of experiments where ammonium chloride is substituted for ammonium tartrate the pH curves are quite similar to the preceding ones. The periodic changes in the curve occur

as before. They are shown in figure 4. Hence, these variations must be due to some factor other than the alternate metabolism of dextrin and the tartrate ion. Here, as in previous experiments, changes in acidity are accompanied by changes in morphology.

Table 8 shows that when ammonium chloride is substituted for ammonium tartrate the medium is negative to Nessler's

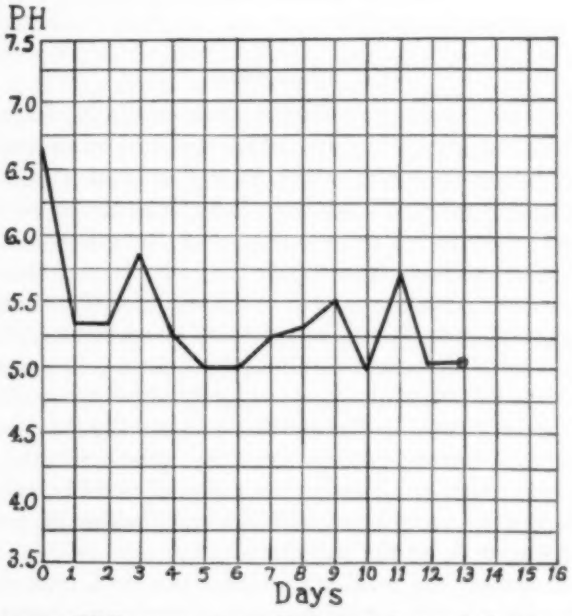

FIG. 4. SHOWING PH PLOTTED AGAINST TIME WHEN B. COLI IS GROWN IN MEDIUM B2 IN WHICH AMMONIUM CHLORIDE HAS BEEN SUBSTITUTED FOR AMMONIUM TARTRATE

reagent. However, after *B. coli* has grown in the media it becomes positive, consequently *B. coli* brings about some change of ammonium chloride which permits the positive test for ammonia. Thus, it is indicated that the ammonia radicle is changed to ammonia before it is utilized by the organism.

After *B. coli* has grown in this medium from five to seven days the test for the tartrate ion becomes positive although it was

originally absent. It must be remembered that the test employed is not specific since a positive test is obtained with any alpha-beta-hydroxy-acid. It would appear possible for dextrin to be converted into tartaric acid by the action of *B. coli* yet it seems quite unlikely that the conversion would take place at such a rate as to cause an accumulation in the media since the tartrate ion is so readily utilized by *B. coli*. Later investigation showed this acid to be an unsaturated compound partaking of the nature of propriolic acid.

DISCUSSION

It has been shown by numerous observers that with time bacteria show variations in form when repeated examinations are made of the organism growing in a particular tube or flask. These changes may be, and have been, interpreted as evidence of degeneration forms or as evidence of the existence of a life cycle. When *B. coli* were studied by the Gram stain over a period of about thirteen days in broth, either with or without the addition of glucose, it was noted that, in addition to the usual abnormal cells, at times Gram-positive granules appeared in the distorted cells, blue-staining or suggestively Gram-positive coccoid forms appeared in the media or many fine bluish-staining (suggestively Gram-positive) granules were evident. It was also observed that at all stages the material could be successfully transplanted to agar slants with the production of good, even luxuriant, growth on the inoculated area.

It was noted that in Koser's media the fecal type of *B. coli* showed within a few hours the almost complete alteration into fine granular forms with practically no normal or abnormal rods present. Transplants from such cultures onto agar slants gave a profuse growth in twenty-four to forty-eight hours.

In order to study the possible relation of the appearance of the various forms to metabolism and hydrogen ion concentration a synthetic medium containing dextrin and ammonium tartrate as the essential nutrient substances was found. It was possible in this medium to trace the metabolism with the accompanying changes in morphology and Gram reaction. During the fourteen day periods of observation it was noted that on one day a culture

may consist principally or entirely of abnormal forms while on a subsequent day there may appear to be present practically only normal forms; frequently this occurred two or three times within the period of observation. Usually in the later period only abnormal forms were noted. Transplantations to agar slants from such abnormal forms always gave profuse growth, suggesting that the abnormal forms were able to multiply and produce typical *B. coli* in the new environment.

The changes of hydrogen ion concentration of the media during the growth of the organism did not apparently bear a definite relation to the appearance of the various forms. With variation of the media, alteration in the initial hydrogen ion concentration, use of glucose in place of dextrin or of ammonium chloride in place of ammonium tartrate, the pH curve and the final pH of the media could be changed but the sequence of forms and final forms were much the same in all conditions.

From the data it may be noted that between the days when the forms of the organisms altered from predominantly irregular (either distorted, partially Gram-positive coccoid or granular) towards a predominantly typical Gram-negative rod that the hydrogen ion concentration remained unchanged or practically so, whereas when the changes moved in the direction of the appearance of irregular forms the reaction of the media showed more or less marked changes.

It was possible to follow readily the utilization of the two important nutritive agents in this medium but it was not possible to show any correlation between the disappearance of either of them or the alternate utilization of either of them and the variation in the morphology of the organism.

It might be assumed that the obtaining of growth after transplantation from cultures showing practically or entirely abnormal forms as well as the alterations noted on succeeding days within a culture from predominance of abnormal forms to predominance of normal forms are due to the growth or multiplication not of the abnormal forms but of the limited number of normal forms which were still present. It is of course impossible with the evidence available to state definitely that this might not be the case where predominance of the abnormal morphological forms

occurred in the flask. However, the fact that with a small inoculum profuse growth was obtained on agar slants when transfers were made from cultures in which the abnormal forms (even the fine granules) were essentially the only ones present, hardly permits of the assumption that it was only from existent normal forms that the new growth occurred. Furthermore the absence of very slight changes in pH of the media during the period that changes from abnormal forms to normal forms took place within a culture does not appear to be compatible with the rapid multiplication of normal forms of bacteria.

Only in the later stages, after the tenth day, did it actually appear that the exhaustion of the medium was to be considered as a factor. Previous to this time however the changes in morphology had been noted.

It appears therefore that the changes in morphology described are probably not to be considered as expressions of death of the bacteria. Possibly they may be viewed only as alteration or adaptation to environment. There is no evidence in these experiments which must of necessity be interpreted as implying the existence of so-called life cycles in these bacteria.

CONCLUSIONS

1. Studies concerning *B. coli* grown in a synthetic medium show alterations of morphology and reaction to gram stain to take place.

2. To a certain extent these alterations appear to be cyclic, since we may note in the culture tendencies toward the predominant appearance of irregular forms subsequent to the presence of typical forms, as well as the appearance of typical forms subsequent to the predominant presence of irregular forms.

3. It has not been possible to correlate these changes in morphology with changes in hydrogen ion concentration, or with the utilization of the ingredients of the media.

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THE DISAPPEARANCE OF THE COLI-AEROGENES GROUP IN NATURAL PURIFICATION PROCESSES AS DETERMINED BY DIRECT PLATE COUNTS

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Received for publication, April 22, 1932

Many attempts have been made in recent years to determine the relative death rates of the two sections of the coli-aerogenes group in surface waters. In the past, such studies have been made by the inoculation of the water sample into a liquid fermentative enrichment medium from which the organisms were isolated and identified. Bacteriologists have realized that the enrichment procedure was open to criticism for such determinations. Ruchhoft *et al.* (1931) have discussed the difficulties and shown the impossibility of interpretation of differential tube results in a quantitative way in a recent paper.

Reliable information is needed on the relative rates of disappearance of the various sections and individual strains that belong to this group to aid in the interpretation of the results obtained by routine water examination and especially to indicate the exact significance and importance of the isolation of *Bact. aerogenes* cultures from surface waters. To this end we have attempted to follow by means of the direct planting ferrocyanide citrate agar medium of Noble (1928), the survival of the various members of the coli-aerogenes group at various distances from the initial source of fecal pollution.

PLAN OF SURVEY

Work on this survey was started in June, 1930, and continued until July, 1931. Samples from the following points, which are shown in figure 1, were collected two to three times each week:

1. Raw sewage, North Side activated sludge plant, Chicago.
2. Plant effluent, North Side activated sludge plant, Chicago.
3. North Branch of Chicago River at Fullerton Avenue, Chicago.
4. Chicago Drainage Canal at Lockport, Ill.

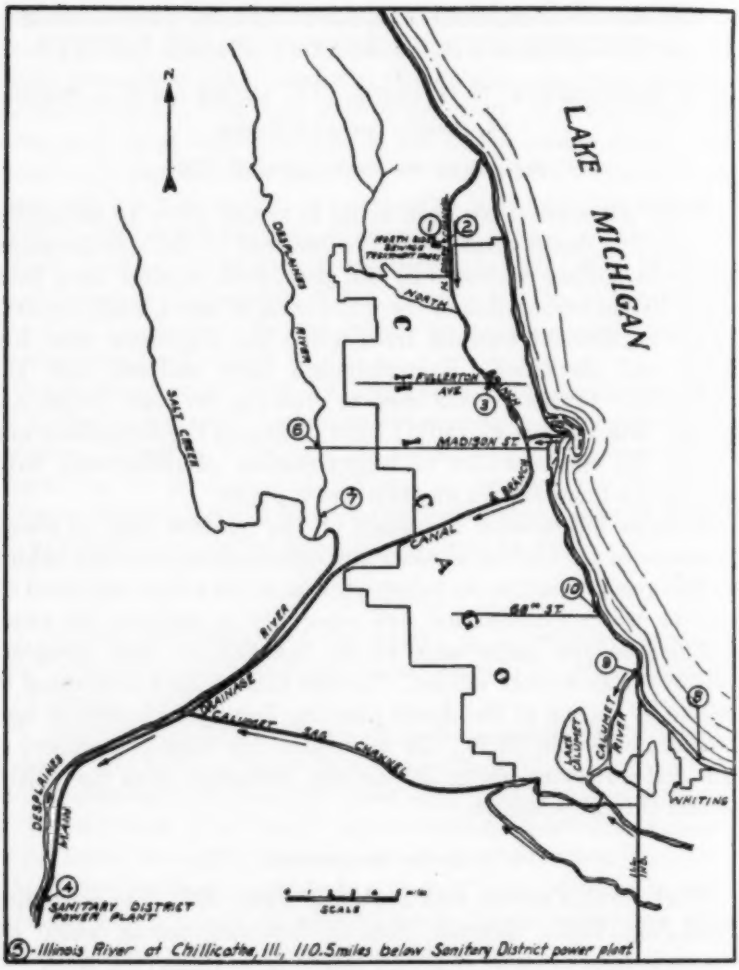
LAKE MICHIGAN
N
NORTH
FULLERTON AVE.
MADISON ST.
CHICAGO
CANAL
BRANCH
CHICAGO RIVER
SOUTH BRANCH
SOUTH FORK
SALT CREEK
98TH ST.
CALUMET RIVER
CALUMET SAG CHANNEL
DES PLAINES RIVER
MAIN
DRAINAGE
LAKE CALUMET
WHITING
SANITARY DISTRICT POWER PLANT
SCALE
5-Illinois River at Chillicothe, Ill., 110.5 miles below Sanitary District power plant

FIG. 1

5. Illinois River at Chillicothe, Ill.
6. Desplaines River at Madison Street, River Forest, Ill.
7. Desplaines River at Riverside, Ill.
8. Lake Michigan at Whiting, Indiana water works intake.
9. Lake Michigan at mouth of Calumet River.
10. Lake Michigan at Chicago, 68th Street pumping station intake.

The samples from the Illinois River at Chillicothe were examined in the Chillicothe Laboratory of the Sanitary District of Chicago and those from the drainage canal at Lockport were examined in our Joliet Laboratory. All other samples were examined in our Main Laboratory at Chicago. All samples were therefore planted within about four hours of time of collection.

MEDIA AND METHODS

All of the media used during this study were prepared in the main laboratory and delivered to the branch laboratories in small batches. Thus, the uniformity of media in all laboratories was assured. To obtain uniformity in procedure and technique each technician was given a short course of training and instruction at the main laboratory. The procedure at each laboratory was to plant all samples in parallel into standard lactose broth and into the Noble medium. The positive presumptive tests obtained in the lactose broth dilution tubes were transferred in parallel to eosine methylene-blue agar plates and into brilliant-green lactose peptone bile fermentation tubes for confirmation. Growth on E.M.B. plates and gas formation in the brilliant-green bile medium was required for positive tests. It is interesting to note that cultures which failed to ferment the brilliant-green bile medium invariably also failed to grow on the E.M.B. plates.

The ferrocyanide citrate agar medium was prepared at the time of planting by the addition of the proper amounts of the necessary chemicals in two solutions to the base agar. This is not Noble's latest procedure[1] but it was the procedure most suitable

[1] In the latest procedure the mixed chemical solution has been modified so that it can be used at once while the old chemical mixture required "ageing" for several days to produce the maximum productivity. We have been using this latest procedure with the freshly prepared "mix" in our main laboratory for the past three months and have found practically no difference in the results obtained with it and with the older procedure.

for use in three laboratories working together on a survey such as ours. In planting Noble's medium wherever 10 cc. portions of water were required quintuplet plates were made. If two dilutions were required triplicate plates of each dilution were made. The dilutions were selected to obtain coli-aerogenes colony counts of 25 to 100 per plate in one of the dilutions.

VARIATION IN COLONY TYPES ON NOBLE'S MEDIUM

Several months after the investigation was started it was found that all *Bact. coli*-like colonies did not exhibit the biochemical characteristics of *Bact. coli*. This was also true for the *Bact. aerogenes* type colonies. Both types of colonies invariably fermented lactose with the production of gas, indicating that they belonged in the group, but were not properly classified by colony morphology. It was also discovered that the so-called intermediate soil type members of the group did not form distinctive colonies on the medium. The majority of these would be mistaken for *Bact. coli* by colony morphology while some resembled *Bact. aerogenes*. Systematic picking of all varieties of colonies from each sample was therefore started and continued to the end of the investigation. The macroscopic colony classification, colony color, size and shape and the biochemical differential reactions used in our previous work were obtained and correlated for 2231 strains during the study.

STATISTICAL TREATMENT

Phelp's indices for the coli-aerogenes group were calculated from the results of the standard procedure confirmed tests for each sample. The average *Bact. coli* and *Bact. aerogenes* type colony counts were obtained for each sample planted on Noble's medium. The ratio of the *Bact. coli* colony count to the *Bact. aerogenes* colony count was calculated for every sample. Monthly average results were calculated for each station. The monthly results were again averaged to obtain one representative result for the thirteen-month period covered by the survey. The results of each month were given equal weight in obtaining final averages.

EXPERIMENTAL DATA

The basic data obtained as described are shown in table 1. We believe that the data on the two groups of organisms given in this table are more reliable than results so far obtained by fermentation tube methods, because of the unreliability of differentiation based on samples from broth tubes, as shown by our work (*loc. cit.*).

TABLE 1

Average coli-aerogenes counts and indices for period of survey

All counts and indices expressed in bacteria per cubic centimeter

SAMPLING STATION	TOTAL NUMBER OF SAMPLES ANALYZED		AVERAGE OF DAILY RATIOS COLI COLONY COUNT OVER COLI-AEROGENES COLONY COUNT	COUNTS OF NOBLE'S FERROCYANIDE CITRATE AGAR 37°C. 44 HOURS			STANDARD METHODS PHELPS' INDICES PER CUBIC CENTIMETER
				COLI COLONY TYPES	AEROGENES COLONY TYPES	COLI-AEROGENES COUNTS	
North Side raw sewage	1	137	1.78	8,271	6,163	14,534	41,240
effluent	2	131	1.37	309	319	628	1,790
North Branch, Chicago River	3	135	2.43	1,076	641	1,717	6,830
Canal, Lockport	4	214	1.49	3,064	3,374	6,438	10,550
Illinois River at Chillicothe	5	156	2.97	25.2	15.6	40.8	56.5
Desplaines River at Madison Street, Chicago	6	72*	1.80	282	388	670	1,080
Desplaines River at Riverside	7	25†	2.21	128	76	207	434
Lake Michigan at Whiting, Ind.	8	142	1.47	5.54	6.4	11.94	43.6
Lake Michigan at mouth of Calumet River	9	147	1.04	2.02	3.18	5.2	13.7
Lake Michigan at 68th Street Crib, Chicago	10	134	0.58	0.106	0.115	0.221	0.495

* Six months survey.

† Three months survey.

The coli-aerogenes counts in table 1 are considerably lower

than the Phelps' Indices. There is quite a variation in the differences between the coli-aerogenes counts on this medium and the Phelps' standard method index. These differences have a tendency to increase with increasing counts. The average numerical results obtained by these two methods are not comparable. These results can not be used in judging the real value of the Noble's medium. Since the Phelps' index is based on an inverse probability analysis there is no reason to expect a direct counting method to check it. A satisfactory comparable study of these two methods is therefore somewhat involved and will be discussed in another paper. For the present let us simply quote Reed (1928) who states

> that the fact of most interest . . . . is the smoothness of the results obtained from the plate counts of stored water in contrast to either the Phelps' index or that of the most probably number, and it seems that this fact constitutes the strongest argument for a plate method. On general statistical grounds it would be expected that a counting process would be superior to the process involving fermentation tubes; *for both the Phelps' index and the most probable number index depend upon a type of inverse probability analysis that makes them much more uncertain than would be any probability or proportion determined through a direct counting process.*

Noble's medium has been experimentally designed to be as productive of *Bact. coli* as of *Bact. aerogenes.* In view of this fact and because the medium is solid the ratio of the representative organisms to each other at the time of planting can be obtained. This is not true for the lactose broth, which as we have previously shown gives very distorted ratios of the initial numbers of organisms. This is due to the abilities of certain strains to outgrow and produce inimical conditions for the development and recovery of other strains that may have been originally present in equal numbers.

The results in table 1 show a remarkable decrease, of similar magnitude, in both the organisms forming the *Bact. coli* type and the *Bact. aerogenes* type colonies during the passage of the sewage through the North Side activated sludge plant. The North Branch of the Chicago River at Fullerton Avenue in Chicago is

less polluted than the Drainage Canal at Lockport, as reflected by the total counts.

Lockport is the point of maximum bacterial pollution of the Des Plaines-Illinois River system (Hoskins *et al.* (1927)). From Lockport to Chillicothe the average time of flow is about five and one-half days. In this section of the river the data indicated that the *Bact. coli* types disappeared at about the same rate as the *Bact. aerogenes* types. This is contrary to the prevailing opinion, which is based on unreliable enrichment tube data, that the *Bact. coli* types die off much faster than the *Bact. aerogenes* types. To summarize the results on the Illinois River, we found that in the six-day flow from the sources of fecal pollution even though there was a 99.36 per cent reduction in the coli-aerogenes count there was very little change in the proportion of these organisms.

The source of most of the pollution of Lake Michigan in the Chicago area is the Indiana Harbor Ship Canal through which sewage from Gary and East Chicago may enter the Lake (Crohurst and Veldee (1927)). The Whiting, Indiana, sampling point was therefore nearest to the source of fecal pollution and the 68th Street Crib at Chicago was the most remote of the lake sampling points included in this survey. The coli-aerogenes indices and counts on the lake points were much lower than those obtained on the Illinois River at Chillicothe, but nevertheless the proportions of the *Bact. coli* type and *Bact. aerogenes* type colonies were very similar to the proportions found on the rivers and in the sewage plant effluent.

An examination of the average daily ratios of the *Bact. coli* type colony counts to the *Bact. aerogenes* type colony counts at the various sampling stations shows that these are quite variable. There is, however, no apparent relationship between these average daily ratios and the magnitude of the pollution indices. The percentages of the *Bact. coli* type count to the coli-aerogenes total count at the various stations also exhibit what at first sight appear to be unexplainable variations. For instance, at Chillicothe in the section of the river where considerable self purification has taken place the *Bact. coli* count formed 61.7 per cent of the group count while in the raw sewage at Chicago the *Bact. coli* count was only 57 per cent of the group count.

APPLICATION OF BIOCHEMICAL TESTS TO COLONY COUNTS OF NOBLE'S MEDIUM

A variation in the colony morphology of the various strains in their passage down river such as may be produced by dissociation might explain the above apparent anomaly. To determine whether this was the case a very careful study of the agreement between the macroscopic colony classification and the biochemical characteristics of the strains at each station was made. The

TABLE 2

Biochemical check of accuracy of the macroscopic interpretation of colonies on Noble's medium at all sampling points

COLONY TYPE ON NOBLE'S MEDIUM	PERCENTAGE OF BACT. COLI COLONIES				PERCENTAGE OF BACT. AEROGENES COLONIES			
	Distribution by biochemical tests							
	Bact. coli	Irregular or mixed	Intermediate	*Bact. aerogenes*	*Bact. coli*	Irregular or mixed	Intermediate	*Bact. aerogenes*
Raw sewage	51.6	3.9	12.5	25.0	11.5	7.7	6.9	72.3
Sewage plant effluent	48.2	7.2	14.5	20.5	14.1	20.6	8.7	52.3
North Branch Chicago River	60.0	6.0	11.6	21.6	12.6	21	3.2	61.5
Canal, Lockport	49.2	11.8	27.3	6.4	14.1	23.4	4.1	51.0
Illinois River, Chillicothe	36	28.3	9.8	18.5	4.9	32.1	8.7	52
Desplaines River	53	9.0	9.0	16.9	9.7	12.9	12.9	61.3
Lake Michigan, Whiting	58.5	9.0	3.3	25.2	6.7	9.0	2.3	78.7
Lake Michigan, mouth of Calumet River	50.5	10.8	10.8	16.2	10.3	11.1	6.8	68.3
Lake Michigan, 68th Street Crib	34.0	4.6	27.2	34.0	11.0	4.9	1.2	81.7

details of this study are beyond the scope of this paper but, in brief, about 100 to 200 colonies of each type for each sampling point were isolated, differentiated, purified and re-differentiated during the study. The net results are summarized in table 2 and indicate a considerable difference, by biochemical classification of the distribution of the macroscopic colony types at the various stations. The results also compensate for any possible differences in colony interpretation at the different laboratories due to the personal equation. The percentage distributions of various

types were applied to the average coli-aerogenes counts obtained at each station, as shown in the following table for the raw sewage sampling station.

	MACROSCOPIC COLONY TYPE		ACTUAL BIOCHEMICAL DIFFERENTIATION			
			Bact. coli	Irregular	Intermediate	*Bact. aerogenes*
Raw sewage	Percentage distribution from table 2	*Bact. coli* colonies	51.6	3.9	12.5	25
		Bact. aerogenes colonies	11.5	7.7	6.9	72.3
	Application of biochemical differential results to macroscopic counts	*Bact. coli* type 8270	4,250	322	1,060	2,060
		Bact. aerogenes type 6260	720	480	433	4,520
	Totals		4,970	802	1,493	6,580

The average counts obtained in this way for all stations are given in table 3. In table 4 the percentages of the *Bact. coli* in the coli-aerogenes group as obtained by the direct count are compared with those obtained after the application of the biochemical differentiation results. The changes in the *Bact. coli* counts from station to station as shown in table 3 are of the same character as those already discussed for table 1. The percentages in table 4, however, indicate better the changes brought about by the application of the biochemical differentiation data to the direct counts. The percentages of *Bact. coli* based on macroscopic counts have irregular fluctuations from point to point. After application of the differential reaction results the inverse relationship between the percentages of *Bact. coli* and the stage of self purification is brought out. The reduction of this percentage is surprisingly small being only about 20 per cent from the maximum point to the minimum point of pollution in our survey. While the coli-aerogenes count was decreased 99.36 per cent between the maximum and minimum point on the Illinois River there was only 8 per cent reduction in the *Bact. coli* percentage of the count.

TABLE 3

Average counts, coli-aerogenes group (application of biochemical differential results at each station)

Annual average—Bacteria per cubic centimeter

	FECAL ORIGIN, BACT. COLI	PROBABLY NON-FECAL ORIGIN			
		Irregular or mixed	Intermediate	*Bact. aerogenes* and *Bact. cloacae*	Total
North Side raw sewage	4,970	802	1,463	6,580	8,845
North Side sewage plant effluent	192	87	73	230	390
North Branch, Chicago River	711	197	142	620	959
Canal, Lockport	1,985	1,148	973	1,916	4,030
Illinois River, Chillicothe	9.86	12.1	3.85	13.8	29.75
Desplaines River, Madison Street	187	75	75	286	436
Lake Michigan, Whiting, Ind.	3.76	1.08	0.33	6.45	7.86
Lake Michigan, mouth of Calumet River	1.35	0.57	0.44	2.51	3.51
Lake Michigan, 68th Street Crib, Chicago, Ill.	0.049	0.011	0.029	0.130	0.17

TABLE 4

Percentages of Bact. coli in coli-aerogenes group (direct count as obtained macroscopically and after application of the biochemical differentiation data)

STATIONS	PERCENTAGE OF BACT. COLI BY MACROSCOPIC COLONY COUNT	PERCENTAGE OF BACT. COLI BY BIOCHEMICAL REACTION
North Side raw sewage	57.0	35.7
North Side sewage plant effluent	49.2	33.0
North Branch Chicago River, Fullerton Avenue	62.5	42.5
Chicago Drainage Canal, Lockport, Ill.	47.6	33.0
Illinois River at Chillicothe, Ill.	61.7	25.0
Desplaines River at Madison Street, River Forest, Ill.	42.0	30.0
Lake Michigan, Whiting, Ind.	46.5	31.8
Lake Michigan, mouth of Calumet River	36.6	27.2
Lake Michigan, 68th Street Crib, Chicago, Ill.	48.0	22.4

This illustrates very well the tremendous changes that may take place in the total group count accompanied by very small changes in the relative numbers of the different members of the group. In other words the death rates of all the organisms in the coli-aerogenes group appear to be very much the same under the natural conditions of self-purification.

TABLE 5

Percentage reduction in coli-aerogenes counts by activated sludge and by natural purification

INDEX OR COUNT USED		PERCENTAGE BACTERIAL REDUCTION PER CUBIC CENTIMETER			
		North Side activated sludge plant	Illinois River, Lockport, to Chillicothe	Lake Michigan, Whiting, Indiana, to 68th Street Crib	Average
Standard methods, Phelps index		96.52	99.42	98.86	98.27
Direct counts on Noble's medium	Coli-aerogenes group	95.67	99.36	98.14	97.72
	Bact. coli colony count	96.26	99.18	98.02	97.82
	Bact. aerogenes colony count	94.90	99.52	98.20	97.54
Direct counts on Noble's medium after correction by biochemical colony differentiation	*Bact. coli**	96.37	99.49	95.94	97.27
	Bact. aerogenes†	96.50	99.25	97.98	97.91
	Intermediate strains‡	95.31	99.58	91.21	95.35
	Irregular strains§	89.15	98.85	98.98	95.66

* Indol + or − MR + VP − citrate −.

† Indol − MR − VP + citrate +.

‡ Indol − MR + VP − citrate +.

§ All other combinations of the above reactions that satisfy the coli-aerogenes classification.

The percentages of bacterial reduction (per public centimeter) for the coli-aerogenes group in the North Side activated sludge plant, the Illinois River and between two points on Lake Michigan have been calculated in table 5, using various indices. This table shows the excellent checks in bacterial reductions between these points regardless of the method of determining the indices.

We conclude from this table that for determining changes in bacterial population of the coli-aerogenes group between points in sewage treatment plants, rivers or lakes it makes little difference whether Phelps' indices by the standard method or group counts of the organisms on Noble's medium are obtained. This table also indicates that there is little difference in the death rates of the different strains in the group.

CONCLUSIONS

1. The direct count of the coli-aerogenes group, by macroscopic observation of colony growth on Noble's ferrocyanide citrate agar, gives results considerably lower than those obtained by use of the Phelps index, based on results of confirmed tests of fermentation tubes according to standard methods. Any method depending on colony counts on a solid medium is more accurate from a mathematical standpoint than a method dependent upon fermentation tube results, and for this reason the ferrocyanide citrate agar may be of more value than the dilution method for determining counts of the coli-aerogenes group in stream pollution studies.

In the studies reported herein there was little difference in the average percentage reductions of the coli-aerogenes group as determined by either method, as shown in the following table:

	SEWAGE PLANT INFLUENT TO EFFLUENT	ILLINOIS RIVER LOCKPORT TO CHILLICOTHE	LAKE MICHIGAN WHITING TO 68TH STREET
Coli-aerogenes direct count, Noble's medium	95.67	99.36	98.27
Standard method	96.52	99.43	97.72

2. The ferrocyanide-citrate agar medium has been proposed as a satisfactory procedure for differentiation of *Bact. coli* and *Bact. aerogenes* by macroscopic appearance of colony growth, without subsequent confirmation by biochemical tests. Very satisfactory coli-aerogenes group counts were easily obtained and no difficulty was experienced with the extraneous organisms that grow

on the medium. Our results, however, indicated that differentiation of the coli-aerogenes types by colony appearance was quite unreliable and that only from 34 to 60 per cent of the so-called *Bact. coli* colonies were actually *Bact. coli* when confirmed by biochemical tests. The percentage confirmation of *Bact. aerogenes* type colonies varied from 51 to 82 per cent.

When the results of macroscopic counts of the two groups were corrected by biochemical confirmatory tests, we found that the percentage of *Bact. coli* decreased from 35.7 to 22.4 per cent of the total coli-aerogenes count in samples of raw sewage and lake water, respectively, but the colony counts without confirmation showed wide and inconsistent variations. Even with confirmation, there was comparatively little difference in the percentage of *Bact. coli* in raw sewage, polluted river water, or slightly polluted lake water.

These results indicate that during self-purification of polluted water there is comparatively little change in the ratio of *Bact. coli* to *Bact. aerogenes*. Although the total coli-aerogenes group decreased more than 99 per cent between Lockport and Chillicothe, in the Illinois River, the ratio of *Bact. coli* to the total actually increased when determined only by macroscopic identification of the so-called *Bact. coli* type of colony, and when further confirmatory tests were applied the percentage of *Bact. coli* decreased only from 33 to 25 per cent between these stations. In view of the considerable self-purification occurring in this stretch of the river, the changes in the coli-aerogenes ratios are relatively insignificant.

3. If both members of the coli-aerogenes group disappear at comparatively equal rates in passing from highly polluted to relatively unpolluted water, there would appear to be no real necessity for differentiation, and no advantage in the direct count except for its greater mathematical precision and shorter time of determination.

Most of the studies that have been made of coli-aerogenes differentiation on the ferrocyanide citrate medium have been made with pure cultures isolated from feces or from soils and grains, and further studied in soils or stumps. So far as we

know, no studies of self-purification of streams, lakes or of artificial sewage treatment have been made similar to the one reported herewith. When the coli-aerogenes groups are studied, in water, under such conditions, there is a disappointing lack of correlation between the degree of pollution and the viability of *Bact. aerogenes*. If no relative changes are to be expected in the ratio of the two groups during self-purification, it would seem to be rather useless to attempt to devise differential tests for examination of polluted waters, particularly if such tests require confirmation by biochemical methods.

The writers wish to acknowledge the helpful suggestions and criticisms of F. W. Mohlman, Director of Laboratories of the Sanitary District of Chicago under whose direction this survey was made.

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SINGLE CELL DISSOCIATION OF ACID FAST BACTERIA*

MYCOBACTERIUM OF AVIAN TUBERCULOSIS; MYCOBACTERIUM OF "RAT LEPROSY"

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Received for publication May 8, 1932

In a recent study (Kahn, 1929) made of singly isolated viable *Mycobacterium tuberculosis* of the H-37 Saranac Laboratory strain it was found that when these single individuals were grown in microdroplets of Long's medium the resultant multiplication did not take place solely by a process of simple fission but underwent a more complicated type of development process, the more important phases of which were cleavage of the rod into three or more ovoid units, subdivision of these units into minute diplococcoid bodies which later became reduced to the state of fine granules. From these granules, extremely small and delicate rods developed. Later these tiny rods, by a process of elongating and thickening became what may be called the mature *Mycobacterium tuberculosis*.

Differences in the staining reaction of some of the components of the cyclic scheme were also noted, and some of these were found to be non-acid fast. The completed life cycle was observed on individuals of the H-37 strain and also upon single organisms from a so-called wild strain of *Mycobacterium tuberculosis* and there seems to be little question as to the sequence of events, at least under our experimental conditions.

It was next thought advisable to extend those studies to individuals of the avian *Mycobacterium tuberculosis* and about a year was spent in an attempt to study the life cycle of this organism

*This study is part of a group investigation being carried on in conjunction with the Medical Research Board of the National Tuberculosis Association.

with varying results. Petroff and his associates (1927, 1929, 1930) then published their studies having to do with the successful dissociation of the A-1 avian *M. tuberculosis* and also the B-1 strain of the bovine organism. Upon applying Petroff's technic for dissociation to the culture of the avian organism upon which we were working we were able to produce the typical rough and smooth colonies, thus confirming Petroff's findings and also the work of Doan (1931). As will be pointed out in the following sections the number of distinct differences existing between the R and S types is striking to say the least, probably more so than is the case among organisms of more rapidly growing species for which dissociation has been shown. In view of this fact, and also because such variable results had been obtained while attempting to study the life cycle of the organism in undissociated culture, it was considered advisable to determine whether the dissociation phenomenon played a rôle in the cycle of the organism and if such ultimately proved to be the case, to consider if there existed any dissimilarity in this cycle according as to whether the organism was in the R or S phase of this development process.

We considered it probable that if dissociation was to be considered as part of the cyclic phenomenon, a culture from a single bacterial cell from a known R colony form should under certain circumstances give rise to some colony offspring of an S character, while the reverse should be the case with single organisms from initially S cultures. Experiments were designed to determine whether such was the case.

TECHNIC

The cultures of the A-1 avian *Mycobacterium tuberculosis* were dissociated originally on plates of Petroff's egg medium and also on the medium of Proskauer and Beck. The criteria which should determine true dissociation will be discussed in detail in a following section. Suffice it to say here that the S colonies are smooth, round, domed and moistly glistening, while the R colonies are flat, rough in contour and configuration and dull in color. When real dissociation does occur it is a distinct and clear cut phenomenon and no difficulty is encountered in distinguishing the R from the S types.

The typical rough or smooth colony was lifted from the plate with a fine sterile platinum spatula and an emulsion made into suitable culture medium. Single organisms were then isolated with the Chambers micromanipulator which we have found to be admirably adapted to the purpose (Chambers, 1922a, b and c) (Kahn, 1922). Prior to this time investigators have used the single cell method principally for the isolation of rapidly growing organisms, proliferating for the most part diffusely in broth. In our studies it was found necessary to develop the method so that the microdroplets containing the single organisms of this slow growing species could be preserved for a long time in order that periodic observations on change in morphology could be made on identical individuals for several days or weeks.

Observations and descriptions made on preparations stained or otherwise, in which large numbers of organisms are encountered, as has been the case heretofore, must be confusing when life cycle studies are attempted. This is unquestionably the case when the usual hanging drop or agar block technic is employed wherein one encounters a number of living bacteria. With slow growing species it is impossible to keep the eye constantly fixed on the same individual for any length of time and if one glances away from the field only for a brief period one cannot be sure of returning to the organism under original observation. Our technic lends itself readily to making the necessary periodic observations on identical individuals and furthermore it is also of value in obtaining pure line cultures from a single organism such as *M. tuberculosis* which is very difficult to induce to grow in a test tube of liquid medium or on a slant of culture medium such as is usually employed for the more rapidly growing single bacteria of other species.

In view of the fact that with this method a slow-growing single cell may be allowed to develop to colony maturity in the microdroplet before it is transferred to the culture tube, a much larger percentage of successful "takes" are procured than would be the case were the single organism planted in or on a suitable medium. Using the older method of direct transfer an experiment was undertaken involving the separate planting of some 80 single human *M. tuberculosis*, and two successful cultures were obtained.

In a second experiment where the single organisms were allowed to form colonies in the microdroplet before making a transfer, 40 per cent positive cultures developed. This appreciably larger percentage was due solely to the fact that the technic employed made it possible to keep the microdroplets containing the single bacterium intact for many days without drying or spreading and thus the development to colony maturity is permitted under optimal conditions. Time is really saved in the end as the possibility of having planted non-viable forms is precluded.

The method of making the micropipettes as well as the manipulation of the pipette holders have been gone into in detail by Barber (1914) and by Chambers concerning their respective apparatus. Suffice it to say that in these experiments the micropipettes were made from soft glass tubing having an inside diameter of about 1.5 mm. The tubing is cut into lengths of some 14 cm., plugged at both ends with non-absorbent cotton, several of these are placed in wide mouth test tubes and sterilized in the hot air sterilizer. The tubing is then made into a capillary pipette by being drawn out in the Bunsen burner. The fine end of the capillary thus formed is redrawn in the flame of a tiny microburner made from a bit of the above mentioned glass tubing. The opening should be compressed until only the smallest possible flame develops. The flame of this microburner may be further regulated by screw clamps. The fine drawn micropipette is bent at a right angle of from about 3 to 5 mm. This is filled with a dilute suspension of the culture and subsequently mounted into the Chambers micromanipulator. A micromanipulator constructed by Dr. George W. Fitz (1931) is also admirably adapted to this purpose.

The new pattern moisture cell (E. Leitz) having an inside diameter of 5 by 2.3 by 1.5 cm. has been found more serviceable for work of this character than the one originally devised by Barber which is higher and wider.

In view of the fact that this technic involves moving the coverslips on the underside of which the microdroplets containing the single bacteria have been isolated and subsequently mounting these coverslips on hollow ground slides, it is necessary to place

an apron of mica on top of the glass moisture cell and upon this apron the coverslip is placed at right angles, instead of parallel to the top of the moisture cell as has been the case heretofore. (See plate 6, fig. 3.) The mica apron has a slot cut into the surface which lies parallel to the rear of the moisture cell and the droplets are isolated on that portion of the coverslip resting just over the slot. No vaseline or other sealing material is used to hold the coverslip in place but the mica apron is sealed to the rim of the moisture cell with sterile vaseline. The importance of properly clearing the coverslips cannot be overemphasized, and the necessarily elaborate technic directed toward this end has already been described by one of us (Kahn, 1929). Before cleaning the coverslips it has been found of great aid to etch with a diamond point a tiny circle at about the center of the coverslips. The droplets are isolated in and about this circle in such a manner as to form the pattern of a cross. Such an arrangement aids materially in finding the microdroplets both in the fresh condition and when the preparation is stained. No. 1, 24 x 50 mm. is the thickness and size of the coverslip employed. It is necessary to handle the coverslips with forceps throughout the technic. The technic for preparing the coverslips has been worked out after considerable experimentation and has been found most satisfactory for removing every trace of foreign material from the coverslips. Unless this is done foreign particles of one kind or another will cling to either surface of the coverslip and it is easy to see how such a state of affairs would be highly confusing to the observer. A number of coverslips are prepared at a time by the above-mentioned process and are held ready for use. Formerly it was thought that a slight trace of grease would prevent the isolated microdroplets from spreading, but this adds the additional hazard of mistaking the small grease particles for fragments of organisms or granules and accordingly was not employed. With the maintenance of proper moisture and temperature there is very little difficulty in keeping the droplet intact. A viscid medium also aids materially in this regard, but agar or gelatin should not be added as the microscopic particles cannot be removed by clarification.

MEDIA TO BE USED

Naturally, the medium to be used varies with the nutritive requirements of the organism. However, successful adaptation to this technic has for one of its prerequisites a fairly viscid substance. For the work pertaining to the avian strains of *M. tuberculosis* and also to the mycobacterium of "rat leprosy" 365 (11) the following formula was employed for the liquid serum medium in which the single cells or small groups were isolated.

Distilled water, 1000 cc.
Lean chopped beef heart, 750 grams.
Simmer at boiling point until bloody appearance disappears.
Make up loss by evaporation.
Filter through cotton wool.
Peptone, 10 grams.
NaCl, 1. grams.
Adjust pH to 7.0.
Ferrous ammonium citrate, 0.025 gram.
Glycerol C.P., 75 cc.
Place in Arnold sterilizer for forty-five minutes at 100°C.
Adjust pH to 7.4 and filter twice through filter paper.

Prior to autoclaving, the medium is clarified by allowing it to run twice through a Sharples clarifier. Sterile inactivated rabbit or human serum is then added in amounts of 7 per cent and after suitable sterility tests have been completed the medium is ready for use.

The actual preparation of the culture from which the single bacterium is to be isolated is of utmost importance. It has been our experience that the youngest cultures are the ones best suited to the purpose as they give the largest percentage of successful single cell cultures in view of the fact that the possibility of isolating non-viable organisms is minimized and that the growth potential is higher than in old cultures. Not alone should the culture actually used be a young one, but the stock from which this plant is made should be induced to active growth by several rapid transfers. This procedure is suited to organisms growing on liquid medium or to undissociated cultures on slants of solid medium. In dissociation work, however, where one desires to obtain a single bacterium from the R or S *colonies* as they occur, the

entire colony must be lifted from the plate and an emulsion directly made of this by gently shaking in a sterile test tube containing a few cubic centimeters of the above mentioned medium and a few glass beads. After letting this preparation stand a few minutes to insure settling of the coarser particles the micropipette is inserted and the tip is broken by gently rubbing against the wall of the test tube and sufficient of the suspension is drawn up to fill the micropipette to just beyond the elbow. Thus, with the moisture chamber, mica apron and sterile cover slip properly mounted, the worker is ready to begin operation and with proper dilution of culture, size of drop, etc., some of the microdroplets will be found to contain a single bacterium or a small group of two to six. No accurate observation of the bacterial population is attempted until the coverslip has been removed from the moisture chamber and mounted on a deep hollow ground slide. The method of accomplishing this has already been described (Kahn, 1929).

After the preparations have been mounted on the hollow ground slide the scratched circle on the coverslip is located with the low power objective and a chart is made of the location of each microdroplet. (These droplets should be about 1 low power field apart and of such a size that the entire droplet is visible under a 10a oil immersion lens.) Each droplet is indicated on the chart by a circle which should be of sufficient diameter to permit the accurate and careful drawing of the single cell or small group of bacteria contained therein. As daily observations were made on the preparations in which a study was being made of the life cycle, considerable time was saved by having made a circular rubber stamp, each circle representing a microdroplet.

With a 10a oil immersion lens and a 10× eye piece each microdroplet is carefully scrutinized and an accurate drawing made of the rod or granule contained. Care must be taken to focus in all depths of the droplet and the edges of the droplet must be carefully scrutinized for a possible microörganism. After the initial twenty-four-hour period of incubation it is essential to conduct all microscopical examinations of the preparations in a hot box having a temperature of from 30° to 32°C. Otherwise,

condensation occurs on the underslide of the coverslip and the preparation becomes flooded.

A droplet containing more than 6 microörganisms was discarded as unfit for accurate observation. Generally a few hours were devoted to making these preparations and from a number a few were selected most nearly approaching the optimal and the remainder discarded.

With practice one finds no difficulty in isolating a considerable number of droplets containing one tubercle bacillus or small groups of two or three bacteria.

As the growth rate of this organism is not sufficiently rapid to make constant or hourly observations practical, such observations were made daily. After each observation the oil is carefully removed from the micro-culture with xylene and the preparation resealed with the hot sealing mixture (Kahn, 1929).

Control and other droplets which were proved to be free of any organism by microscopical examination *invariably remained sterile.* A trained observer has no difficulty in recognizing the presence of organisms when such are present or of the various elements which go to make the cyclic scheme when these are known. No débris or other foreign material is to be observed in the micro-droplets if the medium is properly prepared. The addition of agar or gelatin to the medium makes proper clarification impossible.

MICRO-COLONY OBSERVATIONS AND THE MICRO-COLONY METHOD OF OBTAINING CULTURES FROM A SINGLE BACTERIUM

Aside from applying this technic to the observation of growth phenomenon it may also be used in obtaining relatively rapid information as to the character and nature of a colony in regard to R or S features. As has already been reported by us (Kahn and Schwarzkopf, 1931) the R micro-colony grows in a dense network of coarse fibers while the S micro-colony is delicate and lace-like in structure. The S colonies are also slower growing than are the R. Other differences have been pointed out. Intimate immunological phenomena may also be studied by this method.

Aside from this, however, the technic has one of its principal

applications in obtaining a considerably larger percentage of successful positive cultures from single organisms, especially in the case of the slower growing species. This is accomplished by allowing a microcolony to form in the droplet containing the single bacterium while still mounted on the hollow ground slide and before any attempt is made to transfer to culture tubes.

In the case of those bacterial species which form several generations in twenty-four hours, the resultant colony is usually apparent at the end of a day of incubation or less. Not infrequently a period of lag is noted even among individuals of the more rapidly proliferating bacteria, which may extend for as long as twenty-four hours. Naturally a dead organism is sometimes isolated in the microdroplet but such instances are considerably minimized if one takes pains to prepare a young and vigorous stock culture.

Whenever the smallest colony has formed in the microdroplet from a single bacterium, for instance, an aggregate of some ten to fifteen cells, one is almost certain of obtaining growth upon a slant of medium. The replanting is accomplished in the following manner.

With a small, clean, sharp iridectomy scalpel the sealing material holding the coverslip to the hollow ground slide is carefully cut away. After as much of this as possible has been removed, the partially freed coverslip is gently rotated with two fine pointed dissecting needles (one being held in either hand); until the ends of the coverslip are at right angles to the hollow in the slide. Care should be exercised not to expose the microdroplets by allowing the coverslip to be, even in the slightest degree, moved from over the depression; then the coverslip is quickly lifted with sterile forceps to the mica apron which has been previously mounted on the moisture chamber. The droplets are prevented from drying by having placed hot distilled water in the bottom of the moisture cell, which has, of course, been previously mounted on the mechanical stage of the microscope. A micropipette is then prepared with a bore somewhat coarser than is usually employed for single cell isolations. It is filled beyond the elbow with suitable sterile liquid medium. The micro-colony is then

located under the objective and with the micropipette already in place, a little medium is expelled around the tiny colony to facilitate manipulation. Then, by applying very gentle suction the entire colony is drawn into the micropipette and may be expelled upon a slant of suitable medium. Due to the small bore of the micropipette, considerable resistance is encountered in expelling the fluid contained therein. This is much facilitated by attaching a 25-cc. Luer syringe to the end of the rubber tubing. A small colony visible to the naked eye will soon form on the seeded solid medium and this may be spread with a platinum loop to insure obtaining sufficient material from which to make the desired sub-cultures.

The above in a general way is a résumé of the technic employed. There are probably a number of points, however, which may only be gained from *actual* experience with the apparatus.

R and S colony types

At the outset, in order to prevent confusion, it may be well to recall some of the distinctive features of the typical R and S types as outlined by Hadley (1927). A number of these will be found to conform to the two colony types as they occur in dissociated cultures of the mycobacterium of avian tuberculosis, of the mycobacterium of bovine tuberculosis as Petroff has shown, and of the acid-fast organism isolated by Chapin from a leprous rat and recently dissociated in this laboratory. (This last mentioned organism is known to many American bacteriologists as the mycobacterium of rat leprosy, but it is questionable if the organism is the etiological factor in leprosy disease of that rodent.) Some investigators of the dissociation phenomenon have been content to use as a criterion of dissociation the topography and general condition of the growth membrane of a culture as it occurs on a liquid medium. It is claimed that the S type grows as a thin, continuous delicate film, white to faintly cream in color, while the R type of growth membrane consists of islands upon the surface of the liquid which are raised, the surface sometimes corrugated, of darker color, or pigmented, which do not tend to coalesce until the culture is of considerable age. We do not consider these

characteristics of the growth membrane on liquid media an accurate criterion of dissociation of members of the mycobacterium groups for the following reasons. The S types on fluid culture will sometimes, to a more or less degree, assume the characteristics which some observers consider typical for the R type, while subsequent plating on solid medium will yield numerous, and sometimes a preponderance of, S types. Second, young and vigorously growing R types will often give rise to the delicate, white, continuous growth film while colonies obtained from such growth will yield only the type characteristic of the R morphology. The condition of the growth membrane is also dependent to some extent on the surface and volume of fluid used. In other words, the morphology of the individual colony is obviously of foremost importance as a criterion of dissociation; the appearance of the growth membrane as it occurs upon fluid medium or slant cannot be relied upon.

Since Petroff published his method in 1927, workers with the mycobacterium group have been able to cultivate these organisms on plates in single colonies. This method is of great importance, for, as Doan (1931) has pointed out, it has marked a great advance in our conception of the bacteriology of this group, and for the first time biological investigations on dissociation may be undertaken with *Mycobacterium tuberculosis* on the same basis as with other microörganisms.

The method in brief consists in filtering an emulsion made from a culture through a double thickness of sterile No. 5 Whatman filter paper. The resultant filtrate is plated on egg medium plates in various dilutions. Some of those dilutions will be found of sufficient concentration to give rise to widely separated colonies on the petri plates. For the general details of the technic the reader is referred to Petroff's article (1929).

After Petroff published his results on the successful dissociation of the A-1 strain of the avian *Mycobacterium tuberculosis*, a culture of the undissociated strain was kindly sent to this laboratory at the time the writers were studying two other strains of this organism, namely, one procured from Dr. Elise l'Esperance which had been isolated from a tuberculous chicken and a second one from

the late Dr. Krumwiede, Research Laboratories, New York City Department of Health, known as avian tubercle bacillus strain 216.

Upon applying the technic as outlined by Petroff to these cultures which had been carried for sometime previously on slants of egg medium, it was found that a number of colonies developed which corresponded to the R and S types as noted by Petroff after he had successfully dissociated the A-1 strain of avian *M. tuberculosis*. By applying the single cell technic as previously outlined, successful cultures from one mycobacterium were obtained from the R type of colony of all three strains with little difficulty. The S types, however, presented quite another problem. In a previous communication (Kahn and Schwarzkopf, 1931) we have already shown that in microculture beginning with some fifteen to thirty organisms per droplet, the growth rate of the R is definitely more rapid than the S. This observation has received partial confirmation in the work of Lurie (1928) who demonstrated that virulent *Mycobacterium tuberculosis* proliferates in tissue at a less rapid rate than does the non-virulent organism. Although some 40 per cent of the single R organisms gave rise to successful microculture with subsequent successful gross culture only two successful single cell cultures were obtained with A-1 smooth form after over 100 isolations, while one successful culture from a smooth colony of the L'Esperance strain was obtained after about the same number of isolations and two successful single cell cultures from the smooth colonies of the Krumwiede strain after some forty isolations. In contrast it may be stated that, when some 5 S type bacilli are seeded in the microdroplet, growth takes place in 75 per cent of instances and a microcolony is formed within the minimum time usually employed for this mutant. It is only when a single S cell is isolated that the above-mentioned difficulties are encountered in inducing multiplication. This marked contrast with the R types presents a number of interesting speculations as to why it is that the single S cell has more difficulty in establishing itself especially, as will be shown later, in view of its apparently enhanced pathogenicity to laboratory animals.

In 1920 Churchman separated from a single colony of *B. coli*

two strains, one which grew well in the presence of gentian violet and another which would not grow at all. These two strains were identical by every other bacteriological test known at that time. With the strain of *B. coli* which grew well in the presence of gentian violet when planted by gross methods, single cell studies were made in 1921 by Churchman and Kahn (1921). These showed clearly that *single cell* inoculations of this pure strain would not grow in the presence of the dye (only 1 positive culture out of 148 single cell plants) while about 80 per cent positive results were obtained in the cultures into which single cells of the same strain were inoculated in plain broth, and almost 100 per cent of growth was obtained with groups of about 30 cells inoculated into a series of tubes containing gentian violet broth.

The results which we have obtained in this present study of the growth of avian *M. tuberculosis* S and R strains appear to agree with the suggestion made at that time by Churchman and Kahn that in the growth of some bacteria a phenomenon which was then termed "communal activity" plays a rôle. This would have a direct bearing on the S forms under discussion.

Description of the S form as obtained from a single mycobacterium

The culture grown from a single S organism of the A-1 strain was plated out on 25 plates of Petroff's egg medium according to the technic already outlined, to preclude the possibility of spontaneous dissociation having taken place. The spontaneous S to R dissociation has not been observed in the single cell strains as long as the organisms were kept on Petroff's medium. The colonies as they occur on this medium after six weeks of incubation are from 0.3 to 1.5 mm. in diameter. Little enlargement takes place even after prolonged incubation. In the younger cultures the colonies are dead-white in color assuming a creamy hue after some ten weeks. The colonies are smooth, round, domed, glistenningly moist, compact, regular, seemingly homogenously, dense; no daughter colonies are formed, nor is there an extension of growth at the peripheral base as is the case with the R form. Any further alteration in a true S colony after many weeks of incubation is due to the drying of the medium and is not a matter of growth-continuity as is the case with the R types.

The S colonies go into suspension with great ease in either 0.85 per cent NaCl solution or distilled water, forming a homogeneous type of suspension free of clumps or spontaneously agglutinative particles. As has been shown by us (Kahn and Schwarzkopf, 1931) the mobility velocities of the S forms of this organism are considerably faster than those of the R. For the A-1 strain they average 2.95 micra per second per volt per centimeter as contrasted to 1.53 for the R forms. For the Krumwiede strain the average for the S types was 1.72 micra per second per volt per centimeter while for the R forms it was 3.11. Similar differences have been shown to obtain for R and S types of the B-1 strain of bovine *M. tuberculosis*. These observations have been confirmed by Reed and Rice (1931) using the acid agglutination method of approach.

Microscopic examinations of slides stained by the Ziehl-Neelsen and Krylow acid-fast methods indicate that the S forms retain the carbol-fuchsin somewhat less than do the R. Forms containing intracellular granules are rarer. The extremes of measurement as determined for several hundred S forms from the single cell cultures with the Filer hair micrometer were 3.36μ to 0.55μ in length, the average being 2.04. This is somewhat shorter in general than the R forms, as will be seen. Clumped and filamentous aggregations of organisms are less often seen than is the case with the R culture.

On gross culture (slant of Petroff egg medium) the entire growth gives a moist glistening appearance. As the culture ages it becomes somewhat dryer and wrinkled. This condition may give the false impression of S to R mutation but application of the plating technic to such a culture will soon convince the observer to the contrary.

Description of the R form as obtained from a single mycobacterium

As was the case with the S types, the cultures obtained from the single R organisms were planted out on several plates of Petroff's egg medium and upon none of these 25 plates did an S form of colony develop. Thus, several hundred colonies were observed for varying periods up to eighteen weeks. The R colo-

nies as they occur on these egg medium plates after having been incubated for six weeks are from 1 to 3 mm. in diameter. They are rough, flat, dry, dull, compact and irregular in configuration. At the periphery there is also an irregularity in outline. The color of the six weeks old colonies is about that of old ivory, definitely darker than that of the S types. The picture is that of a nipple surrounded by an areola of somewhat lighter hue. After some nine weeks of incubation the nipple becomes more prominent and assumes an orange coloration while the colony extends at the periphery. The surface inward from the periphery becomes papillated with minute inundations intervening. The nippled area becomes more glossy but material taken from this area and planted upon egg medium plates gives rise only to the R type of colony. Material planted from any other area of the colony also only gives rise to that type of colony. There is the possibility of error in interpreting very young (three to four weeks) R colonies as S colonies. At this time the growth is more glossy than in the older colonies and the central area is also at this time more prominent. Under high power magnification, however, and with careful examination under proper illuminating conditions the dull periphery designating a subsequent R colony may be seen at the base of the colony. Differentiation between R and S types should not be attempted until the plate is at least five weeks old and, preferably, six. After ten to twelve weeks of incubation the orange color in the central nippled area begins to spread while the peripheral growth becomes of a greyer color. The diameter of the colony at this age is 5 to 7 mm. The papillae in the central glossy orange area become much more numerous, but the nippled peak is still distinct although somewhat flattened. At the end of fourteen or fifteen weeks of incubation the colonies may become as large as 10 mm. in diameter; also there is to be seen an extension of the orange colored central area to within about 0.5 to 1.0 mm. of the periphery. The papillae in the heaped up central area spread further toward the periphery, become smaller and more numerous and lose their characteristic waxy luster. This seems to be the end point of growth for the R colony. No further change takes place even after months of incubation save drying and cracking of the colony.

The measurement of several hundred individual rods after staining with the Ziehl Neelsen and Krylow stain revealed an extreme measurement from 5.5μ to 0.5μ. The average was approximately 2.7μ in length. The impression is gained that the rods from the R colonies are on the whole larger or coarser than those from corresponding S colonies of the same age. Beaded forms and those containing the dark-staining (by the Krylow method) intracellular elements seem to be more numerous. As has been already stated the electrophoretic mobility velocity of the R avian forms is considerably slower than that of the S.

The emulsion formed in 0.85 per cent sodium chloride solution or in distilled water is clumpy, coarse, granular or flaky and spontaneously agglutinative. It is not possible to prepare a smooth even type of suspension as with the S colony type, excepting after prolonged shaking with beads in a shaking machine.

Pathogenicity of the R and S single cell strains

As has been reported by Doan (1931) a striking difference exists between the pathological picture produced in chickens and rabbits according to whether the R or S types of the avian *Mycobacterium tuberculosis* are inoculated. The injections were given intravenously. We have confirmed his findings with these single cell strains. In Medlar's (1931) recent studies on the pathology which the avian organism produces when injected intravenously in rabbits, two types of reaction are also described. The first, which he mentions as occurring in his unvaccinated rabbits which as will be seen from the foregoing, is the classical S type of reaction (confirmed by Medlar who has since planted out his avian culture and found it to be an S form) and the second type of reaction, produced with the same avian culture but in rabbits previously vaccinated with subcutaneous doses of human *M. tuberculosis* closely approximates the disease produced when the R form alone is injected. In order to determine the difference in killing time with the R and S types when identical doses of each are employed the following experiment was carried out with thirteen rabbits. While it is realized that this series of animals is a small one, it will be seen that with few exceptions the single cell S strain of avian

M. tuberculosis killed at a more rapid rate than did the R type, although it cannot be denied that some rabbits show great susceptibility to injections of the R mutant as well. Seven rabbits were selected upon which to test the R culture and each animal received 0.5 mgm. of a forty-two day old culture of the single cell strain grown upon Petroff's egg medium plates. The typical R colonies were picked off and suspended in sterile distilled water. The vessel containing this suspension was then vigorously shaken for forty-five minutes in a shaking machine and filtered through

TABLE 1

Rabbits receiving intravenous inoculations with single cell strains of R or S avian Mycobacterium tuberculosis

RABBIT NUMBER	MUTATION TYPE INJECTED	AMOUNT INJECTED	TIME SURVIVED	MUTATION TYPE RECOVERED ON CULTURE
		mgm.	*days*	
47	R	0.5	67	R
67	R	0.5	31	R and S
77	R	0.5	90	R
89	R	0.5	51	R and S
91	R	0.5	92	R
92	R	0.5	28	R and S
93	R	0.5	77	R
63	S	0.5	23	S and R
64	S	0.5	16	S
65	S	0.5	20	S
94	S	0.5	30	S
95	S	0.5	32	S
96	S	0.5	26	S

several thicknesses of sterile cheese cloth to remove the coarse particles. Five-tenths cubic centimeters of the resultant filtrate was then pipetted into small sterile watch glasses, dried over a water bath and weighed in duplicate on a microbalance having a sensitivity of $\frac{1}{400}$ mgm. The injections in all animals were given in the marginal vein of the ear. The animals were fed the usual adequate laboratory ration and each rabbit was kept in a separate cage. At the death of any animal the cage was thoroughly disinfected. Every effort was made to preclude the possibility of cross infection. It will be noted from table 1, that two of these

animals survived for 90 and 92 days respectively, while one survived 77 days, another 67 days and still another 51 days. Two others died within 28 and 31 days. In the case of the animals which survived for 51 or more days, generalized 4+ tuberculosis was noted in each case and the gross pathology impressed us as being so uniform that we will describe these animals as a group. The animals prior to death all showed great loss of weight.

The lungs contained numerous tubercles varying in size from smaller than 1 to 3 mm. in diameter.

Liver: Moderately enlarged with numerous small tubercles and fibrin coated areas.

Kidneys: Both show tuberculous lesions.

Spleen: Enlarged, very dark color: numerous small and large tubercles on surface.

Large numbers of small (1 mm.) tubercles scattered throughout pleura and peritoneum.

Bone marrow of tibia riddled with tubercles.

Axillary and inguinal lymph nodes enlarged and caseous.

Numerous tubercles under skin.

In two rabbits periventricular tubercles were noted.

The organs of each animal were cultured on Petroff's egg medium.

Four gave rise to pure cultures of the R type while rabbits 92, 89 and 67 gave rise to typical S as well as typical R colonies on plates which were seeded with marrow and liver respectively, while the plates from the other organs revealed only the R types. These S mutants from the single cell R strain were found to show the identical pathogenicity of the original S forms. As will be seen from the following data the two animals (nos. 92 and 67) which died within 28 and 31 days (the time usually taken to produce death with the S type of culture) seemingly spontaneously dissociated the R culture and died of an S type of tuberculosis. These animals showed, however, only a small number of S colonies as did the rabbit which died on the fifty-first day after injection. Unfortunately, no accurate counts were kept of this important ratio. These S types, however, were characteristic in every way for the colony when in that stage of its mutation cycle. Their numbers were certainly far less than 1 per cent of the R colonies.

Pathogenicity of single cell S cultures

The method of preparing this culture for injection, including the age of the culture used, was in every way identical with that employed for preparing the R. The six rabbits used to test the pathogenicity of the single cell S type received 0.5 mgm. of culture by the intravenous route. By consulting table 1 it will be seen that all of these animals died within 32 days, the shortest time taken to kill being 16 days. Thus, the average time for death with the S type culture was 24.5 days as contrasted to 62.2 days with the R type.

The gross pathology of the six rabbits which died from the S inoculations and that of the two rabbits receiving the R inoculum, which died after 28 and 31 days, were all practically the same. The most striking feature encountered was the entire absence of *macroscopic* tubercles. The spleen of all of these animals were tremendously enlarged. The lungs revealed congestion and hemorrhagic infiltration with a distinct bloody discoloration. There were no macroscopic tubercles in the bone marrow. Axillary and inguinal lymph nodes were only moderately enlarged. The kidneys revealed no lesions to the unaided eye while the liver was very dark, sometimes almost black, and moderately enlarged. A mottled discoloration of the liver was encountered in two rabbits. No macroscopic tubercles were encountered in this organ. The picture presented was one of a hemorrhagic septicemic process combined with hepatitis.

Cultures made from the organs of these animals showed for the most part a pure culture of S types. There was one exception to this, however. Rabbit 63, which died 23 days after receiving inoculation, yielded one distinct and characteristic R colony on plates made from the liver, while plates made from the other organs revealed only the S types. The R colony thus obtained was, however, characteristic and satisfied the criteria of differentiation.

Thus, it seemed that, in a limited number of cases at least, R to S and S to R mutation of these single cell strains of the avian *Mycobacterium tuberculosis* occurred in the animal body. This confirms the findings of Doan (1931) on non-single cell strains

as regards the former. These tests revealed also that with an identical dose by weight, the S organism kills usually in less time than does the R, but it can by no means be considered that the R types are bereft of pathogenic potentialities. Thomas (1932) has shown that rabbits vary considerably as regards cytological and other constitutional features in their reaction toward the avian and bovine tubercle bacillus. This work would seem to have a bearing on the varying results obtained when inoculations of the R type were made intravenously in rabbits. It seems, however such discrepancies are not so important when the S types are injected, as all of the animals usually succumb after about thirty days.

EXPERIMENTS TO PRODUCE DISSOCIATION OF THE SINGLE CELL R AND S TYPES WITHOUT THE USE OF ANIMALS

No matter how remote the possibility, the objection could be raised that the dissociation of the single cell strains which took place in the animal body (rabbits) might be due to a previously existing infection of the heterologous mutant. To preclude this possibility experiments were undertaken to determine whether or not dissociation could be induced by other means.

For this purpose the single cell strain of the R type avian *M. tuberculosis* was employed.

Effect of reaction of medium

In DeKruif's (1922) pioneer work on bacterial dissociation he found that with *B. lepisepticum* a medium as acid as pH 6.0 retarded the dissociative process, while an alkaline reaction of pH 8.5 often accelerated it. Amoss (1925) reported a pH of 7.8 as the most favorable reaction for the dissociation of pneumococcus while this phenomenon was retarded with an acid medium. Hadley (1927) reports pH 7.8 as the most favorable reaction for dissociating members of the colon-typhoid group. Reed and Rice (1931a) found that a medium of pH 6.5 to 6.8 induced some S to R mutation with a non-virulent bovine *M. tuberculosis*.

Bearing these observations in mind, ten plates each of Petroff's egg medium were prepared at the following hydrogen-ion con-

centrations: pH 5.0, 5.4, 6.0, 6.4, 6.8, 7.0, 7.2. 7.4, 7.8, 8.0 and 8.5. By applying Petroff's filtering technic colonies were induced to grow sufficiently separated so that the morphology of each could be studied when growth occurred. No growth occurred on any of the plates at pH 5.0, and the growth at pH 5.4 was very sparse and insufficient for observation. Fair growth was obtained at pH 6.0 in some of the plates while others showed very scanty colony formation. In our hands the optimal zones extend from pH 6.8 to pH 7.8. In the more alkaline reactions growth was again retarded but a few colonies formed even at pH 8.5. The plates were incubated for a period of twelve weeks, periodic examinations being made as soon as colony formation occurred (three to four weeks with this method) but on none of the plates were S type colonies noted. It may be inferred that alteration of the H-ion concentration within the zones employed did not bring about R to S dissociation with this single cell strain.

Other experiments were then set up whereby suspensions of R organisms were immersed in acid and alkaline fluid media of varying reaction for varying periods and then planted out upon egg plates having a pH of 7.4. No S colonies were induced to form by this process. Plants were also made upon petri dishes of Proskauer and Beck medium containing in addition 1.5 per cent of agar and 10 per cent of sterile inactivated rabbit serum. No R to S conversion took place upon these plates after seven transfers at three-week intervals, the growth on all of the plates being entirely R in character.

Rapid transfer on fluid media rich in normal rabbit serum

Following the work of Soule (1928) and Reed and Rice (1931b) we employed our glycerol broth formula in 300-cc. amounts and also to this added 7 per cent sterile normal inactivated rabbit serum. The single cell R culture was inoculated and transfers made to the same liquid medium at one week intervals. After each transplantation the older culture was plated upon suitable egg medium with the aid of the Petroff filtering technic and examined after growth had taken place for the presence of the S colonies. In these transfers only the youngest, whitest, thinnest

growth membrane was used as inoculum or for planting upon the plates. After the fourth generation, critical examination revealed the presence of a few colonies which were definitely less rough than those on corresponding plates made from cultures taken from slants of Petroff's egg medium. They were small, usually not larger than 1 mm. in diameter, somewhat whiter than the R type, smoother in appearance but still lacking the glistening luster of the typical S form. About the base of these colonies a periphery of irregularly shaped growth occurred which appeared dry and hard. The miscibility of these colonies in normal sodium chloride solution and distilled water was clumpy and R-like in character. When plants from these suspensions were made upon Petroff's egg medium plates, the resultant colony formation was entirely R in character with only a few (less than 1 per cent) simulating the parent form.

After nine generations on the glycerol bouillon rabbit serum medium, the usual 10 plates were inoculated by the Petroff method and on two of these definite S colonies grew up after eight weeks of incubation. Unfortunately no count was made to show the proportion of R colonies to the S colonies thus induced to development. It is certain, however, that the S types comprised considerably less than 1 per cent of the total. Several plate cultures were then made from these S colonies and in each instance a pure culture of the S colony type was obtained. These were entirely characteristic for the organism in the S phase of its mutation cycle according to the criteria already outlined. Two rabbits inoculated intravenously with this second generation S culture in 0.5 mgm. doses died after twenty-six and twenty-eight days respectively, the gross pathology simulating that produced with the single cell strains of the known S type already described.

R to S mutation induced by aging the culture at 37.5°C.

As has already been reported by us (Kahn and Schwarzkopf, 1932) a culture of the so-called mycobacterium of rat leprosy 368, Chapin (1912) was received some years ago from the National Tuberculosis Association. After several transfers on petri plates of Petroff's egg medium it was noted that one of these cultures,

initially R in character, had undergone spontaneous dissociation after having been incubated for several weeks at 37.5°C. Typical round, domed, smooth, moist and glistening S colonies had developed among the erstwhile R colony population. Single cell strains were obtained from both of these colony types and, while these bred true for three or more generations, sooner or later spontaneous dissociation occurred in both instances from these known pure line strains. Typical R colonies grew on plates receiving inoculation with an actual S colony from a single cell S strain while S colonies developed on plates which were seeded with an actual R colony from an R single cell strain. The mutation in the case of this species is somewhat more prolific from S to R but the R to S change may not be termed at all infrequent with this culture of "rat leprae."

The dissociation of these single cell strains took place spontaneously, no especial effort being made to induce it.

It could not be said that starvation was the factor concerned in the initial R to S dissociation of this culture. The colonies on the plate were not crowded and it is logical to assume that there was an excess of food material present. It was felt by us that the aging process must have been involved.

It has been the practice in this laboratory to keep the stock cultures of *M. tuberculosis*, single cell strains and others, on slants of Petroff's egg medium. Three previous generations are kept of each strain and these in duplicate. One set is kept in the incubator while another is placed in the ice box. Transplants are made about every six weeks.

In view of the fact that an aged culture of the mycobacterium of "rat lepra" had shown R to S spontaneous dissociation after long time incubation at 37.5°C., an R culture of the single cell strain of avian *M. tuberculosis* which had been incubated for some eighteen weeks was planted on ten Petroff egg medium plates with the purpose of obtaining widely separated colonies. After six weeks of incubation, one typical S colony developed on one of the plates while four such colonies developed on another plate. Of the remaining eight plates, two were so heavily seeded that the colonies coalesced, making cultural observation impossible, while

on the others only the typical R colony type developed. The S colonies thus dissociated from the R single cell strain by aging were entirely characteristic for the usual S colony type. Subcultures made of these S forms revealed pure culture of the S colony. Two rabbits were inoculated with 0.5 mgm. of an emulsion made of these S colony forms. One of the animals died in twenty days while the other succumbed in twenty-nine days. The picture of the gross pathology was that of the S type of tuberculosis and pure cultures of S colonies were recovered from liver and spleen of both of these animals.

S to R dissociation with single cell S strain

The successful cultivation of these single S cells was, as has already been reported, a singularly time-consuming operation and it was only after a number of attempts that microcolonies developed from the single cell. When these had spread for considerable distance in the microdroplet they were transferred with the micropipette to a slant of Petroff's medium. The growth characteristics of this colony type have been described.

Before any deliberate attempt was made to dissociate this single cell S strain, twenty-five plate cultures on Petroff's egg medium were prepared with the aid of the Petroff filtering technic. When preparing plates by this method with the S forms it is to be borne in mind that, with the more homogeneous suspension which these S organisms form in normal NaCl solution or distilled water, a larger number are filtered through the No. 5 Whatman filter paper, and in order to obtain plates containing widely separated colonies, the filtrate should be diluted to a higher dilution than is necessary for the R types. These plates for the most part contained widely separated colonies although in four of them the seeding was a little too heavy and only part of the plate was useful for observation.

All of these cultures were incubated for a total of ten weeks and periodical examinations were made to determine whether any R type colonies had made their appearance. Such was not the case.

S to R dissociation by animal inoculation

One of the rabbits used for testing the pathogenicity of the single cell S strain (no. 63) died in twenty-three days and although the pathological picture presented was a typical S type of tuberculosis, plates made from the liver of this animal gave rise to two separate and distinct R colony types. These were then emulsified in distilled water and plated out upon ten plates of Petroff's egg medium. The resultant growth yielded only the R colony which satisfied the criteria for the organism in this form.

Three rabbits were given intravenous inoculations of this growth in half milligram doses with the following results:

Rabbit 51 survived 30 days:

Lung: Few small tubercles about 0.5 mm. in diameter.

Spleen: A few minute tubercles peppered about the surface.

No other gross abnormalities could be noted.

Rabbit 47 survived for 67 days when it was killed. The animal lost over 300 grams in weight and the gross pathological picture presented was that of tuberculosis caused by the R type avian *M. tuberculosis.*

Rabbit 48 survived for 32 or 33 days. When the carcass was found it was so badly gas blown that little could be made from the autopsy.

It is apparent that this S to R mutant was variable in its killing properties but this same condition is also apparent with other strains of the R organism. These discrepancies may be due to differences in the constitutional make-up of the animal which Thomas (1932) has unquestionably shown or it may be that in these cases a considerable amount of S antigen is carried over when the S to R mutation takes place, although Rice (1931) has shown with complement fixation tests that the S to R change in bovine *M. tuberculosis* is accomplished by a loss in specific antigen activity. All of their preparations from activated cultures in which the R colony has shown to be predominant, have been shown to be much inferior as antigens to similar preparations made from cultures composed of S types. Yet they find also that antisera prepared from virulent S organisms contain S

specific antibodies in addition to species antibodies which also react with R antigens, and group antibodies detected by preparations made from related acid-fast species. Antisera prepared against R organisms have been found by them to contain species and group antibodies, the former being present in higher concentration than in S antisera.

Reed and Rice (1931) have noted that the S type of a bovine strain with which they worked, proved highly unstable on Petroff's medium. This has also been our experience with the S types of the B-1 bovine strain. In this culture the S to R change takes place with frequency and on some occasions after several transfers and subsequent storage at room or ice box temperature for several weeks, an entire slant, previously seeded with an S colony type, will give rise to only R forms on subsequent replating. The same condition was found to pertain when this strain was seeded upon Lowenstein's medium. The stability of these S bovine cultures may be enhanced, however, if the stock cultures are kept on the ordinary egg yolk media.

With our single cell cultures of the S A-1 avian type, spontaneous S to R changes have not been noted either on plates of Petroff's or Lowenstein's media. We were constantly on the alert for this type of reaction as according to the work of a number of investigators on a variety of organisms such a change is one frequently encountered.

We have been successful in demonstrating the S to R mutation by planting the culture on the medium of Proskauer and Beck or that of Long. After four transfers upon plates of 1.5 per cent agar, into which either of these formulae have been incorporated, some typical R colonies were formed from this known pure line S culture. Petroff medium plates were then made from the parent S culture as a control. These gave rise to only the S type of colony while the R colony types from the medium of Proskauer and Beck which had dissociated from the parent culture upon the fourth transfer revealed only R colonies upon subsequently being plated on Petroff's egg medium.

Attempts to induce dissociation were made by applying fixed suspensions of killed organism to the surface of Petroff's egg

medium plates before inoculations were made with the viable form. These plates were allowed to dry for two days in the incubator and were then seeded with the usual filtering technic. Living R forms were seeded upon plates containing dead S or dead R organisms. No dissociation occurred. Living S forms were inoculated upon plates containing dead S or dead R forms with the same negative result.

SUMMARY AND CONCLUSIONS

Our evidence leads us to believe that the culture offspring of a single mycobacterium of the R or S avian *Mycobacterium tuberculosis* or the R or S mycobacterium of "rat leprosy" will under certain conditions give rise to individuals capable of mutation. In other words, that the potential of dissociation is an inherent property of the single cell of each of these two species, regardless as to whether they are in the S or R phase of their mutation cycle.

The R to S mutation of a single cell R culture of avian M. tuberculosis has been induced by the following:

1. Animal passage.
2. Rapid transfers in glycerol broth containing normal inactivated rabbit serum.
3. Aging the culture in the incubator.

The S to R mutation of a single cell S strain of avian M. tuberculosis has been induced by the following:

1. Animal passage.
2. Rapid transfers on the solid medium of Proskauer and Beck.

These findings confirm the work of Petroff, Doan, ourselves and Reed and Rice with non-single-cell strains and remove the objection that dissociation with this species may be merely a juxtaposition of two separate strains as has been suggested. Single cell strains of the R or S types of the mycobacterium of "rat leprosy" 368 mutate spontaneously in each direction on plates of Petroff egg medium.

These observations substantiate the work of Jordan (1928) on *Bacillus paratyphosus* B, and also the observations of Dawson (1928) on the pneumococcus. Reiman (1925) was able to induce S to R mutation with single cell strains of the pneumococcus but

could not induce the R to S. Amoss (1925) found that a single cell strain of the pneumococcus gave rise to avirulent forms when grown in immune serum broth, bile broth and slightly acid broth. More recently Pinner and Voldrich (1932) have demonstrated the important fact that non-pathogenic *Staphylococcus albus*, *Staphylococcus citreus* and *Staphylococcus roseus* may split off spontaneously from a single cell strain of a pathogenic *Staphylococcus aureus* culture. From the *Staphylococcus albus* strain, which had mutated from the single cell *Staphylococcus aureus* culture, they were able to again induce *Staphylococcus aureus* formation, with the reacquired virulence peculiar to the latter.

In this experiment of Pinner and Voldrich, as well as in the experiments on the pneumococcus alluded to above, the lack or presence of the lethal qualities of the culture seems to have been definitely correlated with type mutations. In our hands the single cell strains of the S or R mycobacterium of rat leprosy 368 were not pathogenic for white rats or rabbits. In these experiments 3 cc. of a heavy emulsion of either R or S types were injected intraperitoneally into the rats and as much as 5 cc. injected intravenously into rabbits without in either instance producing untoward symptoms. These animals were sacrificed after twelve weeks and no lesions were noted which suggested tuberculosis or leprosy of the rodent type.

As far as the pathogenicity of the single cell strains of the R and S types of the mycobacterium of avian tuberculosis is concerned, it may be stated even from our small series of animals, that the R mutant or dissociant is definitely not bereft of tuberculogenic properties. The tuberculosis thus induced has invariably terminated fatally. The average time taken to produce fatal results in rabbits when 0.5 mgm. doses of the R organism were given intravenously was 62.2 days as contrasted to 24.5 days with the S mutant employing identical doses. It cannot be denied, however, that some of the rabbits receiving the R inoculation die well within the time usually taken to produce death with the S mutant. On the other hand, in our series of rabbits receiving 0.5 mgm. doses of the S single cell cultures none of the rabbits have survived as long as some of the animals receiving the R

inoculum. Practically all of the former died within 30 days while some of the animals receiving the R survived 92, 90, 77 and 67 days.

As has been pointed out the type of morbid anatomy produced by the R or S type varies considerably. Animals receiving the R culture, if surviving for sufficient time, show an abundance of macroscopic tubercles in a number of the organs while in the animals dying from tuberculosis induced by injections of the S organism, macroscopic tubercles are seldom noted.

These conditions are found to pertain also to animals receiving S cultures mutated from a single cell R avian M. tuberculosis and R cultures mutated from the S colony type. That is to say, the mutant is in all respects typical for its non dissociated homologue and not for the culture from which it was dissociated.

In interpreting these results we must take into consideration the possibility that the difference in pathogenicity with this species may be more apparent than real. Thomas has unquestionably shown the vast differences in susceptibility of individual rabbits. This may be anticipated, as he has shown, by a detailed study of the cytology of the blood. We have attempted to obviate this difference to some extent by using litter mates in all of our experiments as indicated by the numbers. Doan[1] has shown that a given weight of S culture may show upon count, as many as four times the number of organisms contained in a similar weight of R culture and of course such a condition might account for the difference in pathogenicity, as one set of rabbits would be receiving a much more massive dose than the other. Such, however, has not been the case, as the following work of Doan has clearly shown.

Working with single cell strains of R and S avian M. tuberculosis supplied by us, Doan injected into various laboratory animals 15,000,000 carefully counted organisms from cultures of the R or S type. These cultures were of the same age and also from parent cultures of the same age. The first animal of his series to die was a rooster which had received inoculation of the S culture intravenously. Death occurred 25 days after inoculation. The

[1] Personal communication.

fowl showed marked emaciation, cyanosis and massive microscopic tubercle formation without gross lesions. On sectioning liver, spleen and bone marrow Doan found small discrete tubercles made up of epithelioid cells containing vast numbers of acid fast organisms. The second animal to die in Doan's series was a chicken which had also received intravenous inoculation with the avian S culture. Death occurred on the thirty-fifth day after inoculation. The pathological picture was identical with that of the first chicken. In addition, in both of these chickens, Doan found that the lymphocyte count had fallen to about one per cent while the monocytes were respectively 37 per cent and 66 per cent. There was progressive anemia in both, approximately 2 million red cells at death. Two chickens which received the R organisms at the same time and in the same dosage as in the fowl above mentioned were still alive and without external signs of tuberculosis 71 days after inoculation.

The rabbits in Doan's series survived longer than did ours but this may be accounted for by the difference in the size of the dosage employed, ours being by far the larger. The first of Doan's rabbits died 67 days after inoculation with the counted suspension of S avian *M. tuberculosis*. The cause of death was fulminant, generalized miliary tuberculosis. The spleen was greatly enlarged and weighed 25 grams. One of the R rabbits was killed on the same day for comparison and showed no gross evidence of tuberculosis.

These differences cannot entirely be due to accidental dissimilarity in constitution of the animals. The indication strongly suggests itself that there is also an inherent difference in the pathogenicity of the R and S types of this single cell strain of the avian *M. tuberculosis*. These mutation types also differ in other important characteristics such as in the configuration of the colony, in the electrophoretic mobility velocities, in the miscibility in normal sodium chloride solution or distilled water, in the rate of growth, and, as Richardson, Shorr and Loebel (1931) have shown, in the rate of respiration. Experiments are now under way to determine whether there is also a difference in the life cycle of these mutants of the A-1 strain of avian *M. tuberculosis*.

It is of interest to note that with this species it was not found necessary to add anti-R serum to the liquid medium in order to produce the R to S mutation. Normal serum sufficed. It may well have been, however, that the addition of anti-R serum would have enhanced the process as has been found to be the case with a variety of organisms.

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PLATE 1

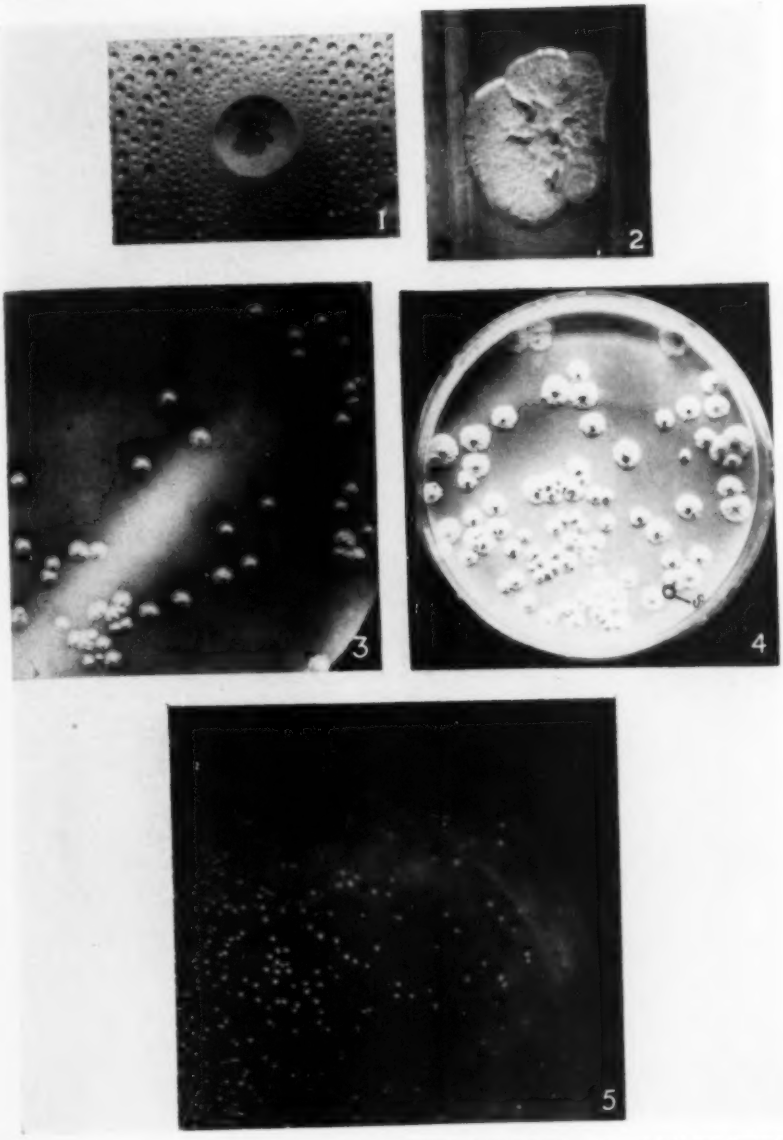
FIG. 1. Microcolony formed from single R Mycobacterium of avian tuberculosis growing in microdroplet of medium.

FIG. 2. Macroscopic colony on slant of Petroff egg medium formed from microcolony in figure 1.

FIG. 3. Pure culture of R colonies on Petroff egg medium plate formed from macroscopic colony in figure 2. Six weeks old.

FIG. 4. Single S colony obtained after injecting single cell R culture intravenously in rabbit. Material for plating obtained from marrow of tibia. Nine weeks old.

FIG. 5. Pure culture of S colonies developing on Petroff egg medium plate. Culture obtained from single S colony in figure 4. Five weeks old.



(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

PLATE 2

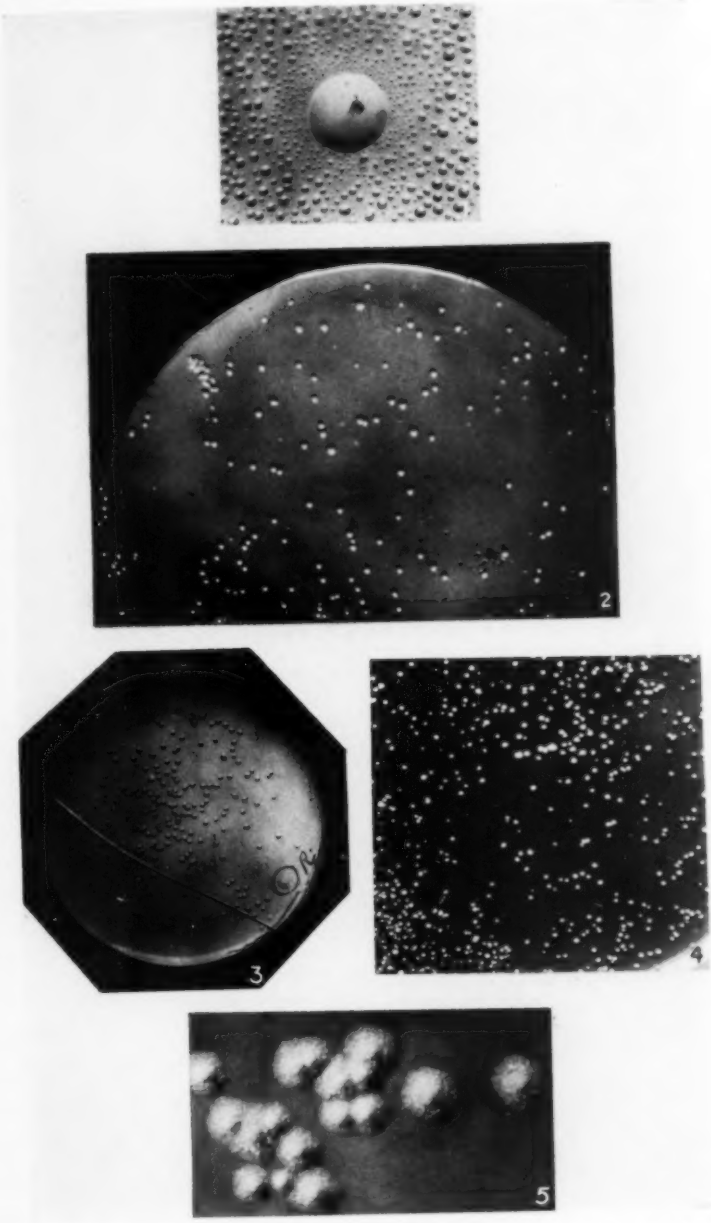
FIG. 1. Microcolony developing from single S Mycobacterium avian tuberculosis growing in microdroplet of medium.

FIG. 2. Pure culture of S colonies developing on Petroff egg medium plate. Culture obtained from microcolony in figure 1.

FIG. 3. Single R colony developing on Petroff egg medium plate after injection into rabbit of S colony suspension from single cell S strain.

FIG. 4. Pure culture of R colonies obtained from single R colony in figure 3.

FIG. 5. R colonies six weeks old (higher power) from single R colony in figure 3.



(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

PLATE 3

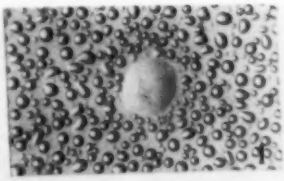
FIG. 1. Microcolony from single R Mycobacterium of rat leprosy developing in microdroplet of medium, slightly out of focus.

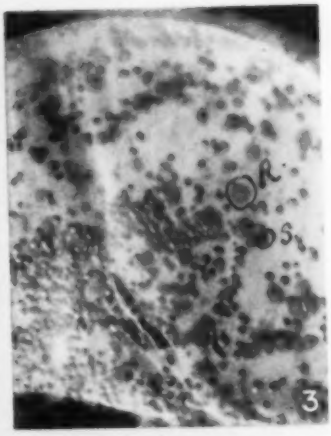
FIG. 2. R type of growth obtained from culture of microcolony in figure 1. Three weeks old.

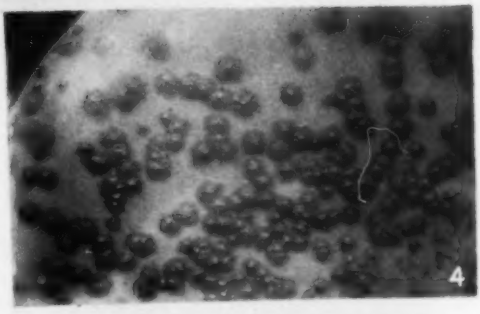
FIG. 3. Spontaneous dissociation Mycobacterium of rat leprosy occurring on plate of Petroff egg medium. Three weeks old.

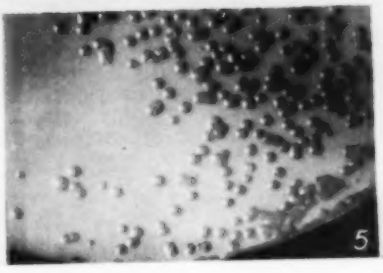
FIG. 4. Pure culture of R type Mycobacterium of rat leprosy obtained from R colony in figure 3.

FIG. 5. Pure culture of S type Mycobacterium of rat leprosy obtained from S colony in figure 3.









(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

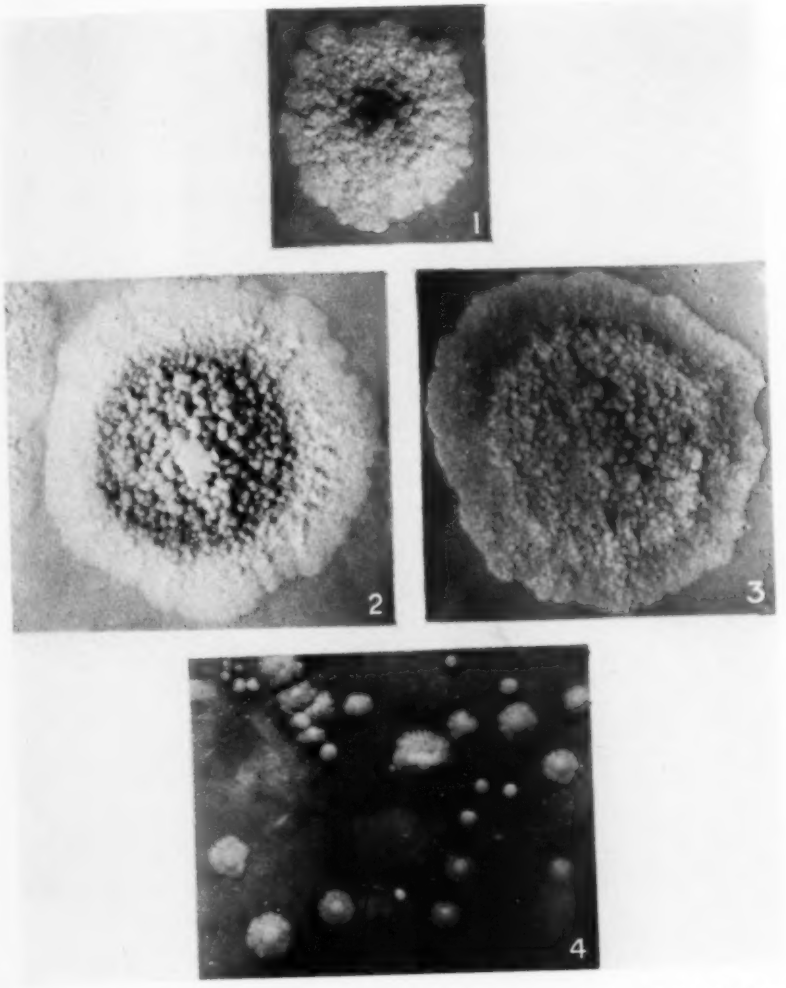
PLATE 4

FIG. 1. Higher magnification R colony Mycobacterium of avian tuberculosis Nine weeks old. Note central orange colored area beginning to develop.

FIG. 2. Higher magnification R colony Mycobacterium of avian tuberculosis Ten weeks old. Note extension of orange colored area toward periphery.

FIG. 3. R colony sixteen weeks old.

FIG. 4. Spontaneous S to R dissociation B-1 strain bovine Mycobacterium tuberculosis occurring on plate of Petroff egg medium. Non-single cell.

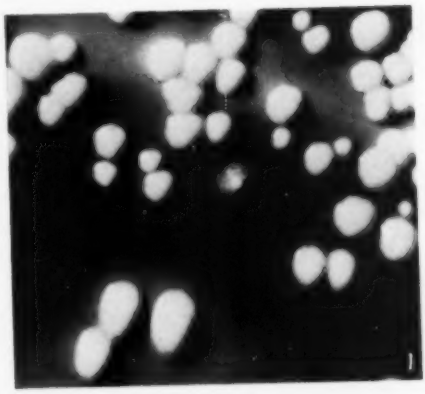


(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

PLATE 5

FIG. 1. R colony developing from single cell S strain Mycobacterium avian tuberculosis after injection into rabbit 63. Material for plating obtained from liver of this animal.

FIGS. 2 AND 3. Intermediate "flat S" or O colony type developing from single cell S strain.



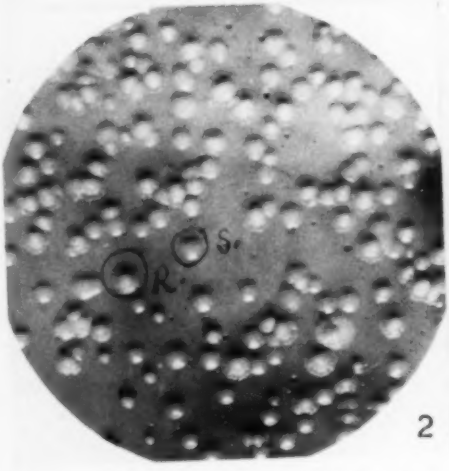
(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

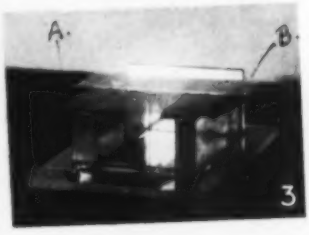
PLATE 6

FIG. 1. Spontaneous S to R dissociation of Mycobacterium of rat leprosy occurring on plate of Petroff egg medium. Single cell S strain employed in seeding this plate. Plate nine days old.

FIG. 2. Spontaneous R to S dissociation of Mycobacterium of rat leprosy occurring on plate of Petroff egg medium. Single cell R strain employed in seeding this plate. Plate twelve days old.

FIG. 3. Moisture chamber used for isolating single cell in microcolony culture technic. Showing relative position of mica apron (*a*) and cover slip (*b*).





(Morton C. Kahn and Helen Schwarzkopf: Single Cell Dissociation of Acid Fast Bacteria)

SOME FACTORS INVOLVED IN THE BIOLOGICAL PRODUCTION OF ACETONE AND BUTYL ALCOHOL[1]

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Received for publication May 26, 1932

The literature on the production of acetone and butyl alcohol by biological agents is an extensive one. Different organisms have been described from time to time which had the ability to form solvents from various raw materials. For an extensive review of the subject the reader is referred to the senior author's doctorate thesis deposited in the Yale University Library.

Early in the present investigation an attempt was made to corroborate the observations of Robinson (1922), namely that, during the fermentation of a simple sugar, such as glucose, in a semi-synthetic medium, acid production by *Clostridium acetobutylicum* follows a definite curve. He concluded from his studies that carbohydrates may be divided into two groups, according to the type of acid production curve which they give; one of these groups produces acid until a maximum is reached, after which the amount of acid in the culture decreases very rapidly, while the other shows no decrease in acidity after the maximum is attained.

It was apparent from the beginning that the bacterial strains which were used in the present work did not behave according to the description of Robinson. The acidity produced from the various sugars studied did not decrease materially after the maximum had been reached. Determinations of the quantities of butyl alcohol and acetone present in the cultures showed the

[1] This paper covers in part the work submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Doctor of Philosophy.

presence of normal yields of acetone, but only very small amounts of butyl alcohol, or none at all.

It seemed highly desirable to inquire further into the conditions that are necessary for the formation of butanol, as well as acetone, from simple sugars when the fermentation takes place in a semi-synthetic medium, and in this way attempt to arrive at a clearer picture of the chemistry involved in the formation of the solvents.

CHEMICAL METHODS

Acetone. This was determined by Goodwin's (1920) modification of the Messinger (1888) method. The following formula was used in estimating the exact amount of acetone in the culture:

$$\frac{(\text{cc. N/5 I} - \text{cc. N/5 thiosulphate}) \times 0.1934}{\text{cc. of sample}} = \text{grams of acetone}$$

Butyl alcohol. When the material to be analyzed was fermented corn mash, the butyl alcohol was determined in the whole culture, according to the method employed by Weyer and Rettger (1927). This involves direct distillation into a Babcock cream determination bottle which is packed to the shoulder with potassium carbonate. The solvents are salted out and allowed to accumulate in the form of an oily layer in the neck of the bottle, where the amount is read off in cubic centimeters or fractions thereof. The following formula was employed in calculating the amount of butyl alcohol present.

$$\frac{\text{cc. mixed solvents} \times 80.64}{\text{weight of corn}} = \text{per cent yield mixed solvents}$$

The resultant figure divided by 1.5 gives the percentage of butyl alcohol in the culture.

When the fermentation was carried out in media other than corn mash, as for instance in Robinson's semi-synthetic medium (1922), the procedure for the determination of the total solvents was the same as for corn mash. The quantity of each solvent was, however, determined by a different set of calculations. After the total solvents were estimated, the amount of acetone in

the mixture was determined by the Messinger method. This gave the amount of acetone in grams. This figure divided by the specific gravity of acetone, 0.792, gave the acetone yield in cubic centimeters which, when subtracted from the total amount of solvents determined by the potassium carbonate method, gave the butyl alcohol yield. This figure was then multiplied by the specific gravity of butyl alcohol, 0.810, in order to convert the butanol into grams.

Reducing Sugars. Reducing sugars were determined by Benedict's quantitative method (1911) and by Somogyi's modification of the Shaffer and Hartmann method (1926).

BACTERIOLOGICAL METHODS

Preparation of corn mash

The chief medium used in this work was a 5 per cent corn mash prepared in the following manner. Fifty grams of corn meal were added to a liter of water and the mixture boiled for fifteen minutes. The loss in weight due to evaporation was then made up with water and the mash tubed and sterilized at 20 pounds steam pressure for one-half hour. This medium was used in preserving all of the stock cultures used in this work, and in the study of the effect of various conditions on the formation of acetone and butyl alcohol from maize.

Preparation of the inoculum

Stock cultures which had been kept in sealed tubes for about a month were used as the source of the inocula for the various experiments. The tubes were opened under aseptic conditions and about 1 cc. of the material transferred to a tube containing fresh corn mash. This tube was then incubated under anaerobic conditions for twenty-four hours at 37°C. After removal from the incubator the contents of the tube were "pasteurized" by heating in a boiling water bath for forty-five seconds. One cubic centimeter of this "pasteurized" material was used to inoculate another corn mash tube which was again incubated under anaerobic conditions for twenty-four hours at 37°C. Ten cubic centimeters of this material served as the inoculum for all cultures

in which volumes of 100 cc. or more of mash or other medium were used. During the work reported in this paper all of the cultures were subjected to "pasteurization" at least once every three weeks.

Methods of anaerobiosis

When the cultures were made in tubes containing corn mash the vacuum jar method of obtaining anaerobic conditions was used. The inoculated tubes were placed in a Novy jar or some other suitable apparatus, which was then sealed with high-melting-point paraffin. The jar was evacuated by means of a vacuum pump. This was done three times, each evacuation, with the exception of the last, being followed by flushing with carbon dioxide.

When cultures were made in large volumes of corn mash, anaerobic conditions were secured by establishing a symbiosis between an aerobe (*Staphylococcus aureus*), and the solvents-producing organism, *Clostridium acetobutylicum*. This was accomplished in the following manner; simultaneously with the inoculation of a culture of *Clostridium acetobutylicum*, a twenty-four-hour culture of *Staphylococcus aureus* was introduced. It was found that this method gave very good results, perfect anaerobic conditions being secured in practically all of the cultures set up, as was evidenced by the rapid rate of progress which the fermentations made in the first twenty-four hours of incubation.

When the culture was made in a medium which was liquid rather than semi-solid, anaerobic conditions were sought by adding filter paper, either pulped or cut into narrow strips. This method was found not to be uniformly reliable, since many of the cultures that were set up according to this procedure did not show evidence of fermentation at the end of twenty-four hours' incubation at 37°C. In instances where satisfactory fermentation could not be obtained by this device the *Staphylococcus aureus* inoculation was resorted to.

Robinson's semi-synthetic medium. This medium had the following composition:

	grams
Monopotassium phosphate	1.00
Magnesium sulphate	0.20
Sodium chloride	0.01
Ferrous sulphate	0.01
Peptone	5.00
Carbohydrate	3.00
Distilled water	1000.00

Strains of *Clostridium acetobutylicum* used in this work:

Strain St—isolated from barley in 1919
Strain K—isolated from putrefying clam in 1924
Strain M—isolated from corn meal in 1924
Strain SO—isolated from garden soil in 1924
Strain B—isolated from barley in 1925
Strain I—obtained from another laboratory and known to be highly developed as a solvents producer.

All of the above-mentioned strains but the last were isolated by Weyer and Rettger (1927) and have since been maintained in this laboratory. They had been kept in sealed tubes for about five years before this work was started. Upon cultivation from the old stock cultures the organisms were found to have weakened considerably in their power of producing acetone and butyl alcohol. After a series of "pasteurizations" and cultivations, however, the ability to form solvents was regained. Checks on the purity of the cultures were kept throughout this work by plating out weekly on malt extract agar and examining colonies microscopically.

MORPHOLOGICAL, CULTURAL AND FERMENTATION CHARACTERISTICS OF CLOSTRIDIUM ACETOBUTYLICUM

A complete study of the morphological, cultural and fermentative characteristics of the strains of *Clostridium acetobutylicum* employed was not made in this investigation. Enough work was done, however, to satisfy the writers that the organisms with which the work was being done were the same as those used by Weyer and Rettger (1927) and that they had not changed in any of the characteristics which they possessed when previously investigated.

PRODUCTION OF SOLVENTS FROM CORN MASH

Ten cubic centimeters of twenty-four-hour "pasteurized" cultures of the six strains of *Clostridium acetobutylicum* were inoculated into flasks containing 150 cc. of 5 per cent corn mash. The corn was inoculated at the same time with 5 cc. of a twenty-four-hour culture of *Staphylococcus aureus* in nutrient broth. After incubation for four days at 37°C. the cultures were analyzed for total solvents and for acetone, and the quantity of butyl alcohol estimated by subtracting the amount of acetone from the total solvents figure. The results are presented in table 1.

The amounts of solvents produced by all of the strains were rather low. This was explained by the cultures having been

TABLE 1

Production of solvents from 5 per cent corn mash

STRAIN	TOTAL SOLVENTS	BUTYL ALCOHOL	ACETONE
	per cent	*per cent*	*per cent*
St	15.70	10.38	5.32
K	14.95	10.08	4.87
SO	14.20	9.39	4.81
M	13.45	9.02	4.43
1	12.95	8.70	4.25
B	12.65	8.47	4.18

kept in sealed tubes for about five years before they were used in this work. The strains had been "pasteurized" only twice during this entire period. As will be seen in the experiments to be described later, the ability of the organisms to produce acetone and butyl alcohol was increased with repeated "pasteurization," and transfer to new medium.

In this preliminary experiment the writers were able, however, to corroborate the statements of various other investigators that butyl alcohol and acetone are produced in a ratio of approximately 2:1 in a 5 per cent corn mash. Strains St and K were found to be the most active producers of solvents. Strain B gave consistently low yields which, even when increased by continuous "pasteurization," did not approach in volume the production of strains St and K.

The rate of acid production from various carbohydrates in semi-synthetic medium

Twenty-four-hour corn mash cultures of strains St and K were inoculated into tall bottles containing 200 cc. of Robinson's semi-synthetic medium. Anaerobic conditions were obtained by the addition of narrow strips of filter paper to the medium. The bottles were inoculated as soon as they had cooled down to 40°C., after removal from the autoclave, in order to minimize the absorption of oxygen. Duplicate 10-cc. samples of the fermenting medium were removed periodically from the bottles by means of

TABLE 2

Rate of acid production of various carbohydrates in a semi-synthetic medium

SUGAR	0 HOUR	6 HOURS	12 HOURS	24 HOURS	36 HOURS	48 HOURS	96 HOURS
Glucose	1.00	1.70	2.18	2.94	3.42	3.27	3.21
Levulose	1.10	2.01	2.68	3.52	3.83	3.67	3.00
Galactose	1.40	2.00	3.07	4.08	4.59	4.78	5.81
Lactose	1.00	1.42	2.05	4.68	4.91	4.73	4.26
Maltose	0.95	1.21	1.68	3.09	4.67	4.49	3.99
Sucrose	0.90	1.26	1.81	4.18	5.62	4.93	4.57
Xylose	0.90	1.51	2.36	3.61	4.45	4.90	4.23
Arabinose	0.80	1.30	1.80	3.18	4.30	4.74	4.19
Starch	1.10	1.31	1.86	3.39	4.88	4.62	4.15
Dextrin	1.00	1.22	1.63	3.74	5.07	4.81	4.59

sterile pipettes. These were titrated directly with N/10 sodium hydroxide, using phenolphthalein as the indicator. Determinations of the acidity were made at the beginning of the experiment and six, twelve, twenty-four, thirty-six, forty-eight and ninety-six hours after inoculation. Only the results obtained with Strain K are given here. Strain St gave practically the same results.

The carbohydrates investigated were glucose, levulose, galactose, lactose, maltose, sucrose, xylose, arabinose, starch and dextrin. All of these, with the exception of xylose and arabinose, were sterilized in pure aqueous solution by autoclaving at 12 pounds steam pressure for ten minutes. The pentoses were sterilized by filtration through a Berkefeld candle. The final concentration of sugar in the medium was 3 per cent.

The results are given in table 2 and in part in figure 1. They differ very markedly from those obtained by Robinson (1922). Figure 1 represents the acidity curves of four representative sugars of the mono-, di- and polysaccharide groups.

From the results presented here, it is not possible to divide the carbohydrates that were studied into groups according to the type of acidity curves which they give. All of the sugars gave evidence of increasing acidity up to the thirty-sixth hour of incubation,

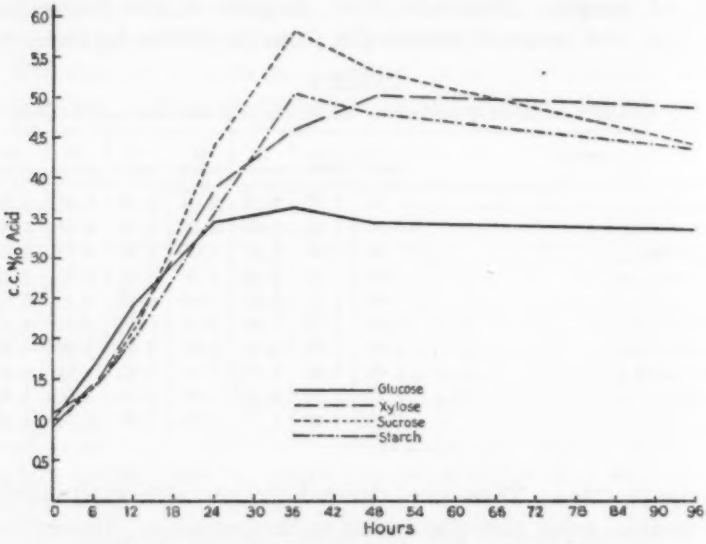

FIG. 1. ACIDITY CURVES OF FOUR CARBOHYDRATES

after which there was some decline in the amount of acid present. However, none of the carbohydrates studied produced the type of acidity curve reported by Robinson. Glucose, fructose, mannose, sucrose, lactose and starch, according to this investigator, yield an acidity curve which reaches a maximum and then declines almost to neutrality at the end of the fermentation. In this investigation none of the sugars gave curves which approached neutrality at the conclusion of the fermentation. The acidity diminished to some extent after the peak had been reached, but

did not fall below 3 cc. of N/10 acid per 10 cc. of culture with any of the carbohydrates.

Determinations of the acidity curves of the other strains were not made, but titrations of the final acidity produced by these strains in the carbohydrates mentioned above showed that a high degree of acidity still persisted when the fermentation, if normal, should have been complete. These results indicated that the abnormal fermentations obtained with Strains St and K were not due to strain peculiarities. The phenomenon in the semi-synthetic medium appeared to be characteristic of all of the strains of *Clostridium acetobutylicum* which were being studied.

The production of solvents from various carbohydrates in semi-synthetic medium

In view of the results obtained in the preceding experiment, an attempt was made to determine what effect the abnormal fermentations had on the production of acetone and butyl alcohol from the carbohydrates which showed high final acidity.

Three per cent of the various sugars studied in the previous experiment were used in Robinson's medium. The medium was subjected to fermentation by all of the strains of *Clostridium acetobutylicum* for four days at 37°C., anaerobic conditions being secured by the use of strips of filter paper. Upon removal from the incubator, acetone and total solvents were determined in all of the cultures. The results are presented in table 3.

Although the different strains produced practically normal amounts of acetone, they formed very little or no butyl alcohol. In no case was more than 2 per cent of this solvent produced, and in many instances no butyl alcohol could be detected. Although the acetone yields are low when compared with the usual powerful fermentation of corn mash, the low values are probably due to the strains having been subjected to "pasteurization" only a few times before they were used in this experiment.

The results obtained here do not agree with the conclusions of Peterson, Fred and Schmidt (1924) that xylose, arabinose and glucose yield butyl alcohol and acetone in a semi-synthetic

medium in a ratio of 2:1. An attempt will be made in the following work to account for this discrepancy.

TABLE 3

Production of solvents from various carbohydrates in semi-synthetic medium

SUGAR	SOLVENT	STRAINS AND PER CENT YIELD					
		St	K	SO	M	I	B
Glucose	Acetone	6.12	5.97	4.97	4.41	4.52	5.02
	Butyl alcohol	2.10	1.35	0	0.93	0.17	0
Levulose	Acetone	5.22	5.11	4.22	4.06	4.03	4.62
	Butyl alcohol	0	0.27	0	0	0	0.60
Galactose	Acetone	5.31	5.02	4.26	3.93	4.00	4.24
	Butyl alcohol	0.70	0	0.13	0	0	0.96
Lactose	Acetone	5.73	5.66	4.43	4.14	4.27	4.87
	Butyl alcohol	0.93	0	0.29	0	1.01	1.02
Maltose	Acetone	6.17	5.91	5.04	4.31	4.49	4.99
	Butyl alcohol	1.94	1.64	1.27	1.20	0	0
Sucrose	Acetone	6.04	5.75	4.65	4.29	4.38	4.72
	Butyl alcohol	0	0.94	1.31	0	0	0
Xylose	Acetone	5.88	5.48	4.70	4.35	4.50	4.89
	Butyl alcohol	0.98	0	0	0	1.37	0
Arabinose	Acetone	5.69	5.40	4.59	4.16	4.51	4.91
	Butyl alcohol	0	0	0.95	0.73	1.09	0
Starch	Acetone	6.21	5.89	5.13	4.48	4.64	4.81
	Butyl alcohol	2.60	1.97	2.06	0	0.17	0.98
Dextrin	Acetone	6.00	5.74	4.82	4.26	4.48	4.73
	Butyl alcohol	0	1.21	1.93	1.14	1.72	0.21
Corn mash control	Acetone	5.62	5.36	5.00	4.87	4.75	4.50
	Butyl alcohol	11.47	10.29	9.89	9.77	9.71	9.23

According to Speakman (1920) and Reilly (1920) butyl alcohol is formed from butyric acid and acetone from acetic acid. The acidity curves obtained in this work show that formation of the acids takes place until a definite maximum is reached, and that

then a slight decline occurs. This fall is probably due to the conversion of the acetic acid to acetone. The failure of the curve to return to an almost neutral point, as it does in a normal fermentation, may be assumed to indicate that conversion of the butyric acid, which is formed from the sugars by the bacteria, does not take place. This assumption is borne out by the data obtained in this experiment and in the acidity determinations (table 2).

The results presented here indicate that the formation of acetone and butyl alcohol are independent chemical processes, since one solvent can be produced in the absence of the other. It is apparent that definite sets of conditions must prevail in the medium in order to induce the formation of one or the other, or of both neutral substances. Up to this point in the investigation no attempt was made to explain the failure of the organisms to produce butyl alcohol. The conditions necessary for the production of this compound have been made the subject of experiments reported in the remainder of this paper.

Production of solvents from various carbohydrate mixtures

It was at first thought that the failure of the organisms to produce butanol in the preceding experiment was perhaps due to the absence of the proper combination of sugars. Consequently twenty-three different carbohydrate mixtures were set up and subjected to fermentation by Strains St and K, at 37°C. for four days, either 1 or 1.5 per cent of each of the different sugars being incorporated, according to the number of sugars employed in the given media. Different combinations of the following carbohydrates were used: glucose, maltose, lactose, sucrose, starch, xylose and arabinose. In each of the 23 mixtures either xylose or arabinose was present, and in one instance both of these pentoses formed a part of the mixture. After removal from the incubator, the cultures were analyzed for acetone and butyl alcohol.

In this experiment acetone was again found to be produced in normal amounts, while butyl alcohol was absent or present in only very small and varying amounts which at best did not exceed

2.61 per cent. The control flask containing 5 per cent corn mash gave a butyl alcohol yield of 13.49. In some of the flasks containing sugar mixtures the production of acetone was rather high as compared with that in the corn mash. No explanation for this fact can be offered. From the data obtained it may be concluded that no special sugar is required for the formation of normal butyl alcohol in a semi-synthetic medium.

Influence of various nutritional and environmental conditions on the production of acetone and butyl alcohol in semi-synthetic medium

Since the type of carbohydrate used appeared to have no apparent influence on the production of butyl alcohol in the semi-

TABLE 4

Influence of peptone concentration on solvents production in semi-synthetic medium

CONCENTRATION OF PEPTONE	SOLVENT	PER CENT YIELD					
		S+	K	SO	M	I	B
per cent							
0.5	Acetone	6.73	5.82	5.03	4.49	4.82	4.73
	Butyl alcohol	0.97	1.21	0.00	0.43	0.23	0.71
1.0	Acetone	6.60	5.96	5.21	4.64	4.88	4.80
	Butyl alcohol	1.27	1.03	0.23	0.37	0.35	0.69
1.5	Acetone	6.94	5.89	5.27	4.68	4.99	4.96
	Butyl alcohol	1.13	1.35	0.19	0.29	0.27	0.00
2.0	Acetone	7.49	5.87	5.33	4.78	5.10	5.16
	Butyl alcohol	0.99	0.00	0.34	0.39	0.00	0.27
Corn mash control	Acetone	7.24	6.43	5.29	5.02	5.11	5.00
	Butyl alcohol	14.12	13.93	11.04	10.20	10.46	9.89

synthetic medium, a study was made of the influence of various other factors on the formation of this solvent. The carbohydrate employed in all of the experiments described in this section was glucose in 3 per cent concentration.

Effect of peptone concentration. Robinson's medium was made up with varying amounts of peptone, namely, 0.5, 1, 1.5 and a 2

per cent Difco peptone. The different flasks were inoculated with one or another of the six strains of *Clostridium acetobutylicum* and incubated for four days at 37°C., anaerobic conditions being secured by the addition of filter paper. As controls, flasks of 5 per cent corn mash were inoculated with all of the strains and subjected to the same procedures as the semi-synthetic medium. The cultures were analyzed for acetone and butyl alcohol; the results are presented in table 4.

Increasing the concentration of peptone did not produce the conditions necessary for the production of appreciable amounts of butyl alcohol. The quantity of acetone formed increases

TABLE 5

Influence of the presence of added colloidal materials on the production of solvents in semi-synthetic medium

ROBINSON'S MEDIUM PLUS	SOLVENT	PER CENT YIELD					
		St	K	SO	M	I	B
Fuller's earth	Acetone	6.21	6.32	5.93	5.77	4.96	4.81
	Butyl alcohol	0.93	1.21	0	1.02	1.34	0.12
Charcoal	Acetone	6.17	6.21	5.97	5.42	5.10	4.79
	Butyl alcohol	1.21	0	0	0.27	0.87	0
Corn mash control	Acetone	7.16	6.52	5.41	5.29	5.13	5.10
	Butyl alcohol	14.22	12.96	10.98	10.72	10.46	10.40

slightly with the increase in percentage of peptone in the medium. This may be due to more complete utilization of the carbohydrate in the presence of a larger amount of available nitrogenous material.

Influence of the presence of colloidal material. Fuller's earth and charcoal were added to Robinson's medium in quantities large enough to give a rather heavy suspension. These media were inoculated with the six strains of *Clostridium acetobutylicum* and incubated for four days at 37°C. As in the previous experiments and in all the work to be reported, 5 per cent corn mash cultures were set up as controls. (Reference to these controls will be omitted in the remainder of this work, with the exception

of inclusion in the tables.) At the conclusion of the incubation period, all of the flasks were analyzed for acetone and butyl alcohol. The results are given in table 5.

The presence of colloidal materials had no visible effect on the fermentation process. As in all of the other experiments reported up to this point, a normal production of acetone occurred, but only very small amounts of butyl alcohol were formed.

Influence of medium as such on solvents production. It was thought that Robinson's semi-synthetic medium may not offer the most suitable conditions for the production of solvents, and may for this reason lead to the formation of acetone only by the

TABLE 6

Influence of medium on solvents production

MEDIUM	SOLVENT	PER CENT YIELD					
		St	K	SO	M	I	B
Robinson's	Acetone	6.37	6.31	6.02	5.86	5.12	5.00
	Butyl alcohol	0	lost	0.72	1.03	0.34	0
Speakman's	Acetone	6.29	6.35	6.04	5.72	5.24	5.09
	Butyl alcohol	1.11	0.63	1.21	0.47	0.69	0
Corn mash control	Acetone	7.08	6.74	5.93	5.89	5.56	5.42
	Butyl alcohol	14.36	13.84	12.06	11.97	11.41	11.07

organisms. A comparative study was made of this medium and that of Speakman. The latter contains, in addition to the substances in Robinson's medium, dipotassium phosphate and manganese sulphate. It has the following composition: dipotassium phosphate, 0.5 gram; monopotassium phosphate, 0.5 gram; magnesium sulphate, 0.2 gram; manganese sulphate, 0.01 gram; ferrous sulphate, 0.01 gram; sodium chloride, 0.01 gram; glucose, 30 grams; distilled water, 1000 cc.

The different strains of the organism were inoculated into flasks containing these two media and incubated at 37°C. for four days. They were then analyzed for acetone and butyl alcohol. The results are presented in table 6.

No differences were apparent in the action of the organisms on

glucose in the two media. Butyl alcohol was not produced in appreciable amounts in either; both seemed to be equally well suited for the formation of acetone. From the data presented, it may be concluded that the mineral composition of the medium has no apparent effect on the production of butyl alcohol. Various investigators have shown that certain minerals are necessary for the growth of the organism; but that these minerals play no part in the formation of the neutral products seems to be borne out by the data presented here.

Effect of neutralization on solvent production. Both the Robinson and Speakman media were found to have an H-ion concentration far below pH 7.0 when they were prepared. It seemed important to determine whether the acidity exerts an inhibitory effect on the organism in such a manner as to prevent the formation of butyl alcohol.

Robinson's medium was prepared according to the formula. A part was neutralized (pH 7.0) with sodium hydroxide; another was adjusted to pH 7.4; while a third was treated with an excess of sterile calcium carbonate. The different strains were inoculated into each of three sets of flasks containing the differently adjusted media, and the flasks incubated for four days at 37°C. Anaerobic conditions were secured by the addition of narrow strips of filter paper. (This procedure was used throughout this work when liquid media were employed.) At the completion of the incubation period all of the cultures were analyzed for acetone and butyl alcohol. The findings are given in table 7. The controls consisted of cultures of the organisms in Robinson's medium, the H-ion concentration of which had not been changed.

The data indicate that the H-ion concentration has a direct effect on the production of solvents. When the medium was alkaline in reaction (pH 7.4) the production of both solvents was entirely inhibited. This is probably due to the organisms themselves being very sensitive to the action of alkalis, as had already been indicated by Weyer and Rettger (1927) in the study of the physiological properties of the bacteria. In the presence of an excess of calcium carbonate no solvents could be demonstrated. The most plausible explanation for this is that the acetic and

butyric acids produced in the fermentation and from which the neutral products are ordinarily formed, are fixed by the carbonate as calcium salts, and are thus prevented from being transformed into the solvents by further bacterial and enzyme action.

In Robinson's medium having unadjusted H-ion concentration, a normal yield of acetone was obtained. Butyl alcohol could not be found in any appreciable quantities, either in this medium or in the flasks that had been adjusted to pH 7.0. The yields of acetone were slightly lower than in the adjusted (7.0) medium.

TABLE 7

Influence of neutralization on the production of solvents in semi-synthetic medium

MEDIUM	SOLVENT	PER CENT YIELD					
		St	K	SO	M	I	B
Robinson's	Acetone	6.28	6.42	6.19	5.72	5.30	5.18
	Butyl alcohol	0.72	0	1.42	1.03	0.19	0.91
R—pH 7.0	Acetone	5.96	5.90	5.83	5.70	4.99	5.02
	Butyl alcohol	0.00	0.32	0.00	0.67	0.00	0.52
R—pH 7.4	Acetone	0.00	0.00	0.00	0.00	0.00	0.00
	Butyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00
R+ $CaCO_3$	Acetone	0.00	0.00	0.00	0.00	0.00	0.00
	Butyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00

R = Robinson's medium.

It would seem then that an initial pH below 7.0 is more beneficial to the organism than a reaction on the alkaline side.

Influence of method of anaerobiosis on solvents production. Up to the present point in the investigation the only method of producing anaerobic conditions in liquid media had been the introduction of narrow strips of filter paper into the flasks in which the fermentation was to take place. In this experiment an attempt was made to determine whether or not the method of producing anaerobic conditions itself exerts any effect on production. Two additional methods were employed. One was that of associated growth of *Clostridium acetobutylicum* with *Staphylococcus aureus*;

the other method involved the use of anaerobic jars which were exhausted by means of a vacuum pump.

All strains of the organism were inoculated into three sets of bottles containing Robinson's medium; one set was incubated in the anaerobic jars; in the second the anaerobic conditions were produced by inoculating the medium with *Staphylococcus aureus*; and in the third the proper oxygen relations were secured by the addition of narrow strips of filter paper. All three sets were incubated for four days at 37°C., and analyzed for acetone and butyl alcohol. The results are presented in table 8.

TABLE 8

Effect of method of anaerobiosis on the production of solvents in semi-synthetic medium

METHOD OF ANAEROBIOSIS	SOLVENT	PER CENT YIELD					
		St	K	SO	M	1	B
Filter paper	Acetone	6.42	6.31	6.20	6.43	5.92	5.61
	Butyl alcohol	1.31	0.97	0.17	1.06	1.17	0
Anaerobic jar	Acetone	6.37	6.29	6.31	6.40	5.84	5.70
	Butyl alcohol	1.12	0	0	0.36	2.13	1.67
Staphylococcus symbiosis	Acetone	6.44	6.21	6.24	6.49	5.80	5.79
	Butyl alcohol	0.63	0	0.99	0.57	1.34	0
Corn mash control	Acetone	7.00	6.81	6.39	6.02	5.93	5.98
	Butyl alcohol	14.31	13.82	13.25	12.47	12.08	12.11

The results obtained in this experiment indicate that the method of producing anaerobic conditions has no effect on the production of either solvent. Butyl alcohol was not produced in appreciable quantities by any of the strains.

Influence of oils on solvents production. Since yellow corn gave normal yields of butyl alcohol, and because it is known to contain as much as 2 per cent oil, it was thought that the oil might play an important rôle in the production of butanol, and, since some investigators have reported isolation of butyl alcohol-forming organisms from this source, maize oil and butter were used in this experiment.

Two sets of Robinson's medium were prepared. To one of these 5 per cent maize oil was added, and to the other 5 per cent creamery butter. Both sets were inoculated with all of the strains of the organism and incubated for four days at 37°C. The results are given in table 9.

An inspection of the data shows that neither of the two oils had any visible effect on the solvents production. As in the previous experiments, normal acetone production was found to take place, but butyl alcohol could not be recovered in appreciable quantities.

TABLE 9

Effect of the addition of oils on the production of solvents in semi-synthetic medium

OIL	SOLVENT	PER CENT YIELD					
		St	K	SO	M	1	3
Maize oil	Acetone	6.61	6.29	6.25	6.32	6.07	5.92
	Butyl alcohol	0.16	1.04	0.00	0.59	0.66	0.00
Butter	Acetone	6.67	6.37	6.29	6.21	5.93	6.01
	Butyl alcohol	0.00	0.45	0.00	1.01	0.94	0.24
Control*	Acetone	6.59	6.29	6.15	6.40	6.19	5.80
	Butyl alcohol	1.23	0.00	1.73	1.02	0.91	0.09

* Robinson's medium without oil.

Influence of addition of various proteins. Since *Clostridium acetobutylicum* is known to be very actively proteolytic, and since good yields of both acetone and butyl alcohol could always be obtained from a 5 per cent corn mash medium, which is rich in protein, an attempt was made to determine the effect of the addition of complex protein materials on the production of solvents in the semi-synthetic medium. The following proteins and protein-rich materials were employed. Pure commercial casein, gluten and egg albumin, sprouted barley, oat seeds, ground roasted peanuts, corn globulin and zein, and wheat gliadin.

The corn globulin was obtained by extracting finely divided yellow corn with 5 per cent sodium chloride solution at 50° to 56°C. for one hour. The material was filtered through filter paper

which had been moistened with 5 per cent sodium chloride solution and heated to 70°C. The filtrate was warmed to 65°C., and allowed to cool gradually in a warm water bath. After standing overnight the crystals of globulin settled out completely. This preparation gave positive biuret, xanthoproteic and Millon reactions.

The wheat gliadin was prepared by converting wheat flour into a thick dough with water. This was kneaded in a stream of cold water until the washings no longer gave a positive iodine test for the presence of starch. The remaining protein was cut into small pieces and extracted with 70 per cent alcohol for twenty-four hours. The extraction was repeated twice. The insoluble material was filtered off and the filtrate evaporated to dryness on a water bath. The substance remaining after the evaporation was recrystallized twice from 70 per cent alcohol. The final product reacted positively to the biuret, xanthoproteic and Millon tests.

Zein was procured from finely ground yellow corn by extraction with ether for twenty-four hours, to remove the maize oils. The residue was extracted with 70 per cent alcohol and the zein prepared by the same procedure as wheat gliadin.

All of these proteins and protein-rich materials were added to Robinson's medium in a concentration of 5 per cent. Flasks of the different media were inoculated with all of the strains of *Clostridium acetobutylicum* and incubated for four days at 37°C. The results are given in table 10.

None of the materials studied, with the exception of zein, gave yields of acetone and butyl alcohol which were at all comparable with those obtained from corn mash. The zein promoted the formation of normal amounts of both solvents in a semi-synthetic medium which, in its absence, stimulated the production of acetone, but not of butyl alcohol. An outstanding characteristic which zein has is its solubility in alcohol.

It was found that barley, gliadin, oat seeds and peanut meats led to the production of much larger yields of solvents than could be obtained in the semi-synthetic medium alone. About one-half of the amount of butyl alcohol found in corn mash fermentations

was recovered in the fermentation of glucose in the semi-synthetic medium to which these substances were added. Since it appeared in these experiments that alcohol-soluble proteins may be necessary for the production of butanol, and since barley contains a pro-

TABLE 10

Influence of the addition of various proteins on the production of solvents in a semi-synthetic medium

PROTEINS	SOLVENTS	PER CENT YIELD					
		St	K	SO	M	1	B
Casein	Acetone	6.74	6.68	6.43	6.02	5.99	5.83
	Butyl alcohol	1.29	0.87	1.05	0.23	0	0.17
Gluten from wheat	Acetone	6.79	6.68	6.50	5.98	6.08	5.87
	Butyl alcohol	1.98	0	0.96	0	1.11	0.45
Gliadin from wheat	Acetone	6.83	6.86	6.40	5.92	6.10	5.85
	Butyl alcohol	7.73	7.41	6.92	6.97	6.48	6.63
Egg albumen	Acetone	6.80	6.72	6.49	6.17	5.91	5.88
	Butyl alcohol	0.52	1.43	0.13	0.99	1.12	0.30
Sprouted barley	Acetone	6.88	6.65	6.54	6.22	6.04	5.91
	Butyl alcohol	6.47	6.60	5.92	5.87	5.96	6.32
Oat seed	Acetone	6.83	6.75	6.40	6.13	6.03	5.80
	Butyl alcohol	8.42	8.21	7.98	7.92	7.12	6.93
Peanut meat	Acetone	6.90	6.64	6.42	6.08	5.98	5.94
	Butyl alcohol	6.14	6.32	5.93	5.71	5.52	6.03
Corn globulin	Acetone	6.71	6.74	6.60	6.07	6.05	5.97
	Butyl alcohol	1.04	1.05	0	1.01	0.49	0.22
Zein	Acetone	6.84	6.79	6.45	5.94	6.04	5.91
	Butyl alcohol	13.98	13.90	13.25	12.10	12.15	12.19
Corn mash control	Acetone	7.12	7.04	6.64	6.02	6.11	5.84
	Butyl alcohol	14.09	14.00	13.82	12.12	12.07	12.01

lamine, hordein, the beneficial action of the different protein complexes was thought to be bound up with the peculiar chemical and perhaps physical properties of alcohol-soluble proteins. In the next experiment an attempt was made to prepare these pro-

teins and study further their influence on the production of butyl alcohol.

Influence of the prolamines from oat seed, barley and peanut meats on solvents production. Although the presence of a prolamine in barley had been a known fact, there was no information at hand regarding its occurrence in oat seeds and peanut meats. Further search of the literature revealed that an alcohol-soluble protein had been isolated from oat seeds and described by Kreusler (1869). Applying the same method as that used for zein, an alcohol-soluble protein was isolated from barley and oat seed without difficulty. The results obtained with peanut meats were not so definite, however. The presence of a prolamine-like substance could be detected in the alcohol extract of peanut meats by means of the xanthoproteic and Millon tests; only faint biuret tests could be obtained, however, and it was impossible to isolate the substance in the same state as the other proteins. The most nearly satisfactory product that could be obtained had the consistency of a gum. Further work on the isolation of this protein from peanuts is still being carried on.

The prepared prolamine or prolamine-allied substances were incorporated in Robinson's medium in 5 per cent concentration. The different prolamine-containing media were inoculated with the six authentic strains of *Clostridium acebutylicum*, incubated at 37°C. for four days and analyzed for acetone and butyl alcohol. The results of the analyses are given in table 11.

All of the alcohol-soluble proteins or protein-like substances studied were found to produce the necessary conditions for the formation of butyl alcohol. The yields of solvents obtained in semi-synthetic media in which these substances were incorporated approached very closely the yields secured in 5 per cent corn mash. Flasks of Robinson's medium to which no prolamine or prolamine-like substance had been added contained normal amounts of acetone but, as has been shown before, no butyl alcohol, or at most very little. All of the data obtained up to this point in the investigation seemed to indicate that an alcohol-soluble protein or allied substance is necessary for the production of normal solvents yields.

The exact rôle which prolamine or a closely allied substance plays in the conversion of carbohydrates to acetone and butyl alcohol is only a matter of conjecture. A discussion of the theories regarding its possible function will be found in the "Discussion" of this paper.

Effect of varying concentrations of prolamine on solvents production. One possible explanation of the action of prolamine or a

TABLE 11

Influence of various prolamines on the production of solvents in semi-synthetic medium

SOURCE OF PROLAMINE	SOLVENT	PER CENT YIELD					
		St	K	SO	M	I	B
Wheat	Acetone	7.09	6.94	6.68	6.16	5.84	5.65
	Butyl alcohol	14.15	13.96	13.04	12.42	11.71	11.55
Corn	Acetone	7.23	7.08	6.86	6.29	5.99	5.94
	Butyl alcohol	14.40	14.08	13.75	12.50	12.11	11.97
Barley	Acetone	7.14	7.03	6.77	6.20	5.87	5.90
	Butyl alcohol	14.21	14.14	13.56	12.43	11.79	11.94
Oat seed	Acetone	7.09	7.12	6.72	6.28	6.01	5.85
	Butyl alcohol	14.16	14.31	13.39	12.62	12.10	11.59
Peanut meat	Acetone	7.11	7.05	6.74	6.19	5.92	5.86
	Butyl alcohol	14.13	14.00	13.56	12.22	12.03	11.84
Robinson's medium without prolamine	Acetone	6.99	7.03	6.71	6.25	5.93	5.82
	Butyl alcohol	1.56	1.16	0.97	0.65	1.03	0.84
Corn mash control	Acetone	7.21	7.10	6.83	6.21	6.04	5.91
	Butyl alcohol	14.24	14.35	13.79	13.55	12.00	12.03

prolamine-like substance is that it may function as a catalyst in the reaction which takes place when butyl alcohol is formed. In order to test this hypothesis a study was made to determine the effect of varying concentrations of prolamine on the production of solvents. The alcohol-soluble protein used was zein. This substance was incorporated into Robinson's medium in flasks in concentrations of 0.25, 0.50, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 per cent.

The flasks were inoculated with all of the stock strains of the organism and incubated for four days at 37°C. At the end of the incubation period acetone and butyl alcohol determinations were

TABLE 12

Influence of various concentrations of zein on the production of solvents in Robinson's semi-synthetic medium

CONCENTRATION OF ZEIN	SOLVENT	PER CENT YIELD					
		St	K	SO	M	I	B
per cent							
0.00	Acetone	7.14	7.06	6.74	6.25	5.92	5.68
	Butyl alcohol	1.09	0.93	0.23	1.16	0.84	0.75
0.25	Acetone	7.08	7.12	6.71	6.19	5.96	5.58
	Butyl alcohol	2.21	2.08	1.97	1.90	1.82	1.73
0.50	Acetone	7.12	7.14	6.70	6.30	6.00	5.72
	Butyl alcohol	3.47	3.21	3.32	3.16	3.41	3.04
1.00	Acetone	7.17	7.09	6.81	6.26	5.91	5.60
	Butyl alcohol	6.35	6.21	6.00	5.95	5.92	5.92
1.50	Acetone	7.03	7.13	6.77	6.28	5.97	5.72
	Butyl alcohol	7.61	7.63	7.54	7.48	7.52	7.41
2.00	Acetone	7.14	7.04	6.83	6.31	5.89	5.69
	Butyl alcohol	9.52	9.61	9.49	9.32	9.11	8.97
3.00	Acetone	7.16	7.08	6.91	6.19	6.04	5.79
	Butyl alcohol	12.92	12.71	12.83	12.45	12.51	12.33
4.00	Acetone	7.07	7.13	6.70	6.14	5.95	5.91
	Butyl alcohol	14.18	14.21	13.82	13.60	12.15	12.00
5.00	Acetone	7.18	7.17	6.79	6.28	5.99	5.84
	Butyl alcohol	14.22	14.28	13.82	13.63	12.13	11.94
Corn mash control	Acetone	7.15	7.19	6.80	6.34	6.11	5.98
	Butyl alcohol	14.21	14.32	13.80	13.61	12.10	11.99

made on all of the cultures. The results of the analyses are presented in table 12.

An inspection of the data shows that the production of acetone is independent of the percentage of zein, while the quantity of

butyl alcohol formed appears to depend directly on the amount of prolamine in the medium. With increasing amounts of zein, increasing amounts of butanol were demonstrated. A concentration of between 4 and 5 per cent prolamine seems to be optimum, however, for the production of maximum quantities of this solvent. This evidence should exclude from consideration the hypothesis that prolamine or a prolamine-like substance plays the rôle of a catalyst in the reaction, since the action of a catalyst, within limits, is not directly dependent on its concentration in the medium in which it is acting.

Influence of the removal of carbohydrate on solvents production. The results obtained in the experiment just reported might lead to the conclusion that the butyl alcohol was derived from the zein itself, rather than from the carbohydrate in the presence of prolamine. The following experiment was set up to prove or disprove this possibility. Five per cent zein was added to one set of flasks containing Robinson's medium from which the glucose was omitted. Another set contained glucose, but no zein. As controls, flasks containing Robinson's medium plus glucose and 5 per cent zein were used. All of the strains of the organism were inoculated into the three different media and incubated at 37°C. for four days. The results are given in table 13.

The organisms failed to produce any solvents in the absence of carbohydrate, no acetone or butyl alcohol being demonstrable in the flasks that contained only zein. When both zein and glucose were present, practically the same amounts of solvents were produced in the semi-synthetic medium as in a 5 per cent corn mash medium. In the absence of the prolamine, normal quantities of acetone were formed from the carbohydrate, but only negligible amounts of butyl alcohol. The data secured in this experiment seem to prove that the neutral products are derived from the carbohydrate, and not from the protein.

Production of solvents from xylose, arabinose and glucose. Since a method of producing solvents in maximum amounts in a semi-synthetic medium now seemed to be at hand, an attempt was made to corroborate the conclusions of Peterson, Fred and

Schmidt (1924) that xylose, arabinose and glucose are fermented, with the formation of the same products in practically the same ratios. Three per cent of each of these carbohydrates, sterilized by filtration through a Berkefeld candle, was incorporated in Robinson's medium to which had been added 5 per cent zein. The different media were inoculated with the six strains of the solvents-producing organism and incubated at 37°C. for four days. The amount of sugar present in the media at the completion of the incubation period was determined, using the methods of Benedict and Somogyi. The results are given in table 14.

TABLE 13

Influence of carbohydrate on the production of solvents in semi-synthetic medium

ROBINSON'S MEDIUM PLUS	SOLVENT	PER CENT YIELD					
		St	K	SO	M	1	B
0.00 per cent glucose + 5 per cent zein	Acetone	0.00	0.00	0.00	0.00	0.00	0.00
	Butyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00
3 per cent glucose + 0 per cent zein	Acetone	7.10	7.18	6.07	6.28	6.15	5.88
	Butyl alcohol	1.23	1.06	0.98	0.47	1.11	0.88
3 per cent glucose + 5 per cent zein	Acetone	7.12	7.22	6.98	6.39	6.08	5.97
	Butyl alcohol	14.34	14.20	13.79	12.56	12.22	12.04
Corn mash control	Acetone	7.08	7.13	6.92	6.44	6.15	6.03
	Butyl alcohol	14.20	14.31	13.93	12.92	12.28	12.15

The analyses showed that each of the carbohydrates yielded acetone and butyl alcohol in a ratio of approximately 1:2. The total yield of solvents from the pentoses was slightly less than from the hexose. The pentoses and hexose were completely destroyed. These results are in exact agreement with those reported by Peterson and his co-workers, who carried out their fermentations in Robinson's synthetic medium *without* the addition of zein. The explanation for their normal yields of solvents may be found in their having introduced enough zein in the inoculum which they used, 10 cc. of a twenty-four-hour culture in 6 per cent corn mash, to allow the fermentation to proceed nor-

mally. When a culture is not highly active, it is very probable that not very much of the zein is destroyed in the first twenty-four hours of fermentation.

Robinson (1922), in his study of the acidity curves produced by the butyl alcohol organism, showed that only 53 per cent of the xylose was destroyed. That Peterson and his group secured 100 per cent utilization of the pentose without the addition of zein would indicate that the conditions prevailing in their fermentation mixtures were different from those obtaining in the fermentations studied by Robinson. This investigator appar-

TABLE 14

Production of solvents from xylose, arabinose and glucose in semi-synthetic medium containing 5 per cent zein

STRAINS	XYLOSE			ARABINOSE			GLUCOSE		
	Acetone	Butyl alcohol	Sugar destroyed	Acetone	Butyl alcohol	Sugar destroyed	Acetone	Butyl alcohol	Sugar destroyed
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
St	6.95	13.81	99.83	6.98	13.99	99.86	7.21	14.19	99.92
K	6.70	13.32	100.00	6.80	13.74	99.45	7.24	14.28	100.00
SO	6.43	12.89	98.91	6.65	13.22	99.72	6.81	13.71	100.00
M	5.92	11.71	100.00	6.00	12.09	99.89	6.21	13.55	99.89
1	5.77	11.59	99.42	5.91	11.74	99.94	6.11	12.21	99.63
B	5.46	10.92	98.90	5.64	11.32	99.63	5.82	11.85	100.00
Robinson's medium without zein; St.	6.84	0.88	54.31	6.98	1.09	58.65	7.16	1.31	61.32

ently did not determine the amount of solvents present in his cultures. If he had done so, it is probable that he would not have found butyl alcohol. This theory is supported by the writers' observation that in Robinson's medium which contained xylose, arabinose and glucose, but no zein, only from 50 to 60 per cent utilization of the carbohydrate took place. These cultures were found to contain normal amounts of acetone, but practically no butyl alcohol. When zein was present in a concentration of 5 per cent in the medium, however, complete utilization of the carbohydrate took place, and yields of butyl alcohol closely approximating those secured from 5 per cent corn mash were obtained.

Influence of the addition of zein to corn mash. Zein, prepared according to the method already described, was added to flasks containing 5 per cent corn mash in a concentration of 5 per cent. Flasks were inoculated with each of the six strains of *Clostridium butylicum* and incubated at 37°C. for four days. Anaerobic conditions in the flasks were secured by inoculating, simultaneously with the Clostridium, from a twenty-four-hour culture of *Staphylococcus aureus* in extract broth. The results of the analyses are given in table 15.

Increasing the amount of zein above the percentage normally present in yellow corn has apparently no effect on the amount of butyl alcohol and acetone produced. The yields from cultures

TABLE 15

Effect of the addition of zein on the production of solvents in 5 per cent corn mash

MEDIUM	SOLVENT	PER CENT YIELD					
		St	K	SO	M	1	B
Corn mash + 5 per cent zein	Acetone	7.04	7.11	6.84	6.26	6.09	6.00
	Butyl alcohol	14.18	14.36	13.89	12.44	12.22	12.13
Corn mash control	Acetone	7.10	7.21	6.90	6.32	6.15	6.04
	Butyl alcohol	14.24	14.38	13.92	12.72	12.41	12.19

containing added zein and from controls containing only the normal amount of the prolamine are approximately the same. It would appear, then, that butyl alcohol is not derived from zein or any other alcohol-soluble protein or closely allied substance.

Influence of the removal of zein from corn mash. In order to determine whether or not there were any other substances in corn which could bring about the production of butyl alcohol in the absence of zein, an attempt was made to remove all of the zein from maize. Finely ground yellow corn was extracted twice with ether to remove the oil, which had already been shown to play no part in the production of butanol. The residue was treated with 80 per cent alcohol until the corn had apparently lost all of its color. This required five or six extractions, each lasting twenty-four hours. The extracted corn was then made

up into a 5 per cent mash, inoculated with the solvents-producing organism, and incubated for four days at 37°C. The results of the analyses are given in table 16.

The cultures in corn from which the prolamine or prolamine-allied substances had been removed yielded normal amounts of acetone, but only small quantities of butyl alcohol. The butanol was obtained, however, in larger amounts than from Robinson's semi-synthetic medium alone. It is quite probable that not all of the zein had been removed. Control cultures of corn mash from which the bulk of the prolamine had not been removed revealed the presence of normal amounts of both solvents. This experiment furnishes added evidence that an alcohol-soluble pro-

TABLE 16

Effect of the removal of zein on the production of solvents from 5 per cent corn mash

MEDIUM	SOLVENT	PER CENT YIELD					
		St	K	SO	M	I	B
Alcohol-extracted corn	Acetone	7.09	7.18	6.80	6.31	6.14	6.00
	Butyl alcohol	3.96	3.89	3.10	2.92	2.83	2.44
Corn mash control	Acetone	7.15	7.18	6.82	6.27	6.10	5.96
	Butyl alcohol	14.42	14.21	13.75	12.71	12.29	12.04

tein or a substance closely associated with it is necessary for normal butyl fermentation.

Influence of the addition of sugar solutions to maize culture. Since it had been found that carbohydrates could not be fermented by *Clostridium acetobutylicum* in a semi-synthetic medium, with the production of normal quantities of butyl alcohol, and that the addition of a prolamine in a concentration of approximately 5 per cent stimulated the formation of this solvent, a study was made of the effect of adding pure sugar solutions to corn mash, which apparently contains optimum quantities of prolamine. Glucose, xylose and starch were the carbohydrates employed. Three per cent solutions of these substances in water were used in making up a 5 per cent corn mash; that is, instead of adding the corn to the water, as was usually done, the mash was made up in the three

per cent solution of the sugar. The different media were inoculated, and incubated for seven days at 37°C. The longer incubation period was thought necessary because of the large amount of carbohydrate present in these media as compared with ordinary corn mash. The results of the analyses are presented in table 17.

From the evidence obtained here it seems apparent that solutions of pure carbohydrates undergo a normal fermentation in the presence of corn mash. Yields of solvents approximating those obtained from ordinary corn were secured from cultures in which the corn had been incorporated in sugar solutions instead of in

TABLE 17

Effect of the addition of carbohydrates on the production of solvents from 5 per cent corn mash

MEDIUM	SOLVENT	PER CENT YIELD					
		St	K	SO	M	I	B
Corn + 3 per cent glucose...	Acetone	7.26	7.30	6.92	6.44	6.28	6.07
	Butyl alcohol	14.48	14.75	14.00	12.97	12.45	12.23
Corn + 3 per cent xylose....	Acetone	6.87	6.74	6.51	6.31	6.04	5.88
	Butyl alcohol	13.77	13.68	12.97	12.74	12.21	11.84
Corn + 3 per cent starch....	Acetone	7.19	7.26	6.99	6.63	6.52	6.29
	Butyl alcohol	14.51	14.68	14.11	13.31	12.98	12.68
Corn mash control.....	Acetone	7.13	7.20	6.90	6.31	6.12	5.92
	Butyl alcohol	14.21	14.51	13.92	12.55	12.31	11.73

water. The total amount of solvents produced in cultures which contained added carbohydrate was greater than that obtained from corn mash alone. However, approximately the same percentage yields were obtained in both types of media. The percentage of each solvent was calculated on the basis of the amount of fermentable carbohydrate present in the medium. The ratio of butyl alcohol to acetone in every case was found to be approximately 2:1. This experiment serves to corroborate the results obtained in the study of the effect of the presence of zein on the fermentation of glucose, xylose and arabinose in a semi-synthetic medium, where it was found that the solvents were produced in approximately the same ratio as from yellow corn.

Acid hydrolysates of certain natural or raw materials as possible sources of the solvents

Various investigators have been interested in the fermentation of the acid hydrolysates of various natural substances, with a view to commercial application. Among the most notable work in this field is that of Fred and his associates, whose main efforts were devoted to the production of lactic acid by *Lactobacillus pentoaceticus*. A review of the work on this subject is included in the senior author's Master thesis deposited in the Yale University Library.

Preparation of the acid hydrolysates. The following substances were subjected to hydrolysis in the present investigation: cotton seed hulls, peanut husks, and oak, chestnut, maple and birch sawdusts. These raw materials were treated in flasks containing 4 per cent sulphuric acid in the proportion of one part of dry material to two parts of the acid. One set of the flasks was autoclaved at 20 pounds steam pressure for two hours, and another at the same pressure for four hours. After removal from the autoclave the contents of the flasks were filtered and the residue leached several times with water to remove the soluble sugar. The leachings and the filtrates were then combined and adjusted with sodium hydroxide to pH 7.0. Determinations were made of the amounts of reducing sugar present in the hydrolysates, Benedict's and Somogyi's methods being employed. The results of the analysis are given in table 18.

Good yields of sugar were procured in the hydrolysis of all of the materials which were used here. The corn cobs gave the largest amounts of reducing carbohydrate, and peanut husks the smallest. The prolongation of the heating over a period of four hours seems to destroy some of the sugar that is secured in the first two hours of heating.

Fermentation of acid hydrolysates with Clostridium acetobutylicum. The sugar solutions obtained in the hydrolysis of the various raw materials with dilute sulphuric acid for two hours at 20 pounds steam pressure were diluted with water until approximately 3 grams of carbohydrate were present in each 100 cc. of solution. They were adjusted to pH 6.6, monopotassium and

dipotassium phosphates being used. One per cent peptone was added. The various carbohydrate solutions were inoculated with strains St and K. The cultures were incubated for four days at 37°C., anaerobiosis being established by inoculation with a cul-

TABLE 18

Amounts of reducing sugar produced in the acid hydrolysis of various natural substances

RAW MATERIALS	TIME OF HYDROLYSIS	PERCENTAGE SUGAR
	hours	
Cottonseed hulls	2	17.54
	4	22.72
Peanut husks	2	8.93
	4	6.44
Douglas fir sawdust	2	16.65
	4	13.20
White pine sawdust	2	13.28
	4	11.38
Maple sawdust	2	18.87
	4	14.94
Birch sawdust	2	21.11
	4	21.50
Chestnut sawdust	2	14.58
	4	11.06
Oak sawdust	2	13.00
	4	10.70
Corn cobs	2	33.32
	4	25.13

ture of *Staphylococcus aureus*. The results of the acetone and butyl alcohol determinations are given in table 19.

Acetone was formed in amounts slightly smaller than in the fermentation of corn mash. The percentage recovery of this solvent approximates closely that obtained in the fermentation

of pure xylose and arabinose in a semi-synthetic medium, with or without the presence of an alcohol-soluble protein. This finding suggests the possibility that the carbohydrate material present

TABLE 19

Production of solvents from the hydrolysates of various natural materials by Clostridium acetobutylicum

HYDROLYSATE	SOLVENT	PER CENT YIELD	
		St	K
Cottonseed hulls	Acetone	6.82	6.89
	Butyl alcohol	1.06	0
Peanut husks	Acetone	6.91	6.96
	Butyl alcohol	1.23	0.75
Douglas fir sawdust	Acetone	6.71	6.65
	Butyl alcohol	0.45	0.92
White pine sawdust	Acetone	6.74	6.82
	Butyl alcohol	0.87	0
Maple sawdust	Acetone	6.47	6.36
	Butyl alcohol	0.21	1.06
Birch sawdust	Acetone	6.56	6.64
	Butyl alcohol	1.17	1.32
Chestnut sawdust	Acetone	6.84	6.90
	Butyl alcohol	0	0.28
Oak sawdust	Acetone	6.15	6.22
	Butyl alcohol	0	0.67
Corn cobs	Acetone	6.88	6.80
	Butyl alcohol	1.36	1.15
Corn mash control	Acetone	7.15	7.22
	Butyl alcohol	14.41	14.38

in the various hydrolytic products is essentially pentose in nature. Only negligible amounts of butyl alcohol could be obtained in the fermentation of the different hydrolysates. This was to be expected, since it had already been shown that the fermentation of

pure sugars in a semi-synthetic medium does not lead to the production of butanol. The medium in which the fermentation was allowed to proceed in this experiment was essentially a mixture of salts, carbohydrate and peptone, no prolamine or prolamine-like substance, which is apparently necessary for the formation of butyl alcohol, being present.

In order to determine whether the acid hydrolysis residue itself contained any hydrolysable substance or substances which would favor butyl alcohol production, if present in the *Clostridium acetobutylicum* fermentation mixture, the residue left after hydrolysis with 4 per cent sulphuric acid was further digested, this time with 80 per cent H_2SO_4 in the cold. By this process cellulose was hydrolyzed, and a considerable amount of reducing carbohydrate obtained. Fermentation experiments were conducted with both the solution and residue, incorporating them separately in the solvents fermentation cultures which contained no corn mash. The results were essentially like those shown in table 18. Little or no butyl alcohol was formed.

Finally, the unhydrolyzed raw materials were subjected to the acetobutylicum fermentation process. The different substances were made up into 5 per cent mashes, and 1 per cent peptone added. They were inoculated with strains St and K of the solvents-producing organism and incubated for four days at 37°C. in association with *Staphylococcus aureus*.

Neither acetone nor butyl alcohol could be detected in the fermentation flasks. This would indicate that the carbohydrate material present in the raw materials is available for fermentation by *Clostridium acetobutylicum* only after it has been converted into simpler forms.

DISCUSSION AND SUMMARY

A study of the morphological, cultural and fermentation properties of the six strains of *Clostridium acetobutylicum* which were used in this investigation showed that they did not differ materially from the original descriptions of Weyer and Rettger (1927). Storage in sealed tubes for a period of about five years had apparently no effect on them, except that their power to produce solvents was diminished. The original ability to ferment carbo-

hydrates with the production of acetone and butyl alcohol was regained quite rapidly by a series of alternate "pasteurizations" and cultivations. Long before the conclusion of this investigation all of the strains had developed into high solvents producers.

The acidity produced in Robinson's semi-synthetic medium containing glucose, levulose, galactose, xylose, arabinose, sucrose, lactose, maltose, starch or dextrin furnished plotted curves which were abnormal when compared to those described by Robinson (1922), in that they all continued on a high level after an early rapid rise, whereas those of Robinson showed a marked drop after the peak. A conclusion that the failure to return to neutrality bore a direct relationship to the failure to produce appreciable amounts of butyl alcohol was, it seemed, justified. The correlation corroborates Speakman's theory that the neutral products (solvents) are formed from the corresponding acids, acetic and butyric.

Numerous attempts showed that butyl alcohol could not be produced in Robinson's semi-synthetic medium as such. Consequently, a study was made of the influence of various factors on the formation of this solvent. It was found that hydrogen-ion concentration, method of anaerobiosis and type of medium used (aside from the occurrence of prolamines or closely related or associated substances), as well as the presence of oil and the concentration of peptone employed in the basic medium, had no visible effect on the amount of butyl alcohol produced from glucose, when this sugar was incorporated into the semi-synthetic medium.

An investigation of the effect of various types of protein on the fermentation showed that the alcohol-soluble proteins from corn, wheat, oats, barley and peanut meats, when used in a concentration of from 4 to 5 per cent, influenced the fermentation in such a way that large yields of butanol were produced. Five different theories suggest themselves as possible explanations for the favorable action of the prolamines or prolamine-allied substances.

1. The prolamine[2] *is the source of butyl alcohol.* Since the

[2] The word prolamine is used in the following discussion to indicate real prolamine, or a prolamine-like substance, or some agent which is chemically or physically bound up closely with prolamine.

amount of butanol produced was found to vary directly with the concentration of alcohol-soluble protein in the medium, up to 4 or 5 per cent, it was at first thought that this material might be the source of the butyl alcohol. This hypothesis was disproved, however, by showing that in the absence of fermentable carbohydrate there was a complete absence of both acetone and butyl alcohol. Further evidence against this theory was obtained when, on the addition of the alcohol-soluble substance to corn mash, which already contains an alcohol-soluble protein, zein, the yield of solvents was not increased above the normal amount.

2. The prolamine acts as a catalyst. The possibility of an alcohol-soluble protein or a substance closely bound up with it acting as a catalyzing agent in the reaction that is involved in the change from carbohydrate to butyl alcohol was discarded when it was shown that the production of butyl alcohol bears a direct relationship to the amount of prolamine present in the medium. A concentration of from 4 to 5 per cent was found to be optimum. The action of known chemical catalysts, is, within limits, not dependent on their concentration.

3. The prolamine is necessary in the metabolism of Clostridium acetobutylicum. The theory that a prolamine is necessary in the metabolism of the solvents-producing organism seems to be a most plausible explanation, in so far as the production of butyl alcohol is concerned. However, prolamines do not appear to be necessary for the production of acetone, or for the bacterial growth itself. They, in all probability, affect the metabolism of the organism in such a way as to favor the production of the enzyme or enzymes necessary for the conversion of butyric acid into butyl alcohol. Such an hypothesis seems quite plausible, in view of the conclusions of various investigators that the type of medium used to grow bacteria has an important effect on the type of enzymes which the organisms produce.

4. The prolamine acts purely as a physical agent. The alcohol-extracts, when incorporated in Robinson's semi-synthetic medium, were found to become very gummy in character. This property of the substance may have a favorable effect on the growth of the organism, particularly since it is a strict anaerobe.

5. The prolamine acts through combined chemical and physical effects. It would seem that the formation of butyl alcohol in the presence of an alcohol-soluble protein or some substance closely connected with it can not be explained purely on the basis of its action on bacterial metabolism, or on its physical effects. Both factors, acting in combination and at the same time, may furnish an explanation of the production of butyl alcohol in the presence of a prolamine, and the almost complete absence of this solvent in media which do not contain this type of protein.

Since it had been shown that *Clostridium acetobutylicum* was unable to produce normal yields of both solvents from a semi-synthetic medium which did not contain a prolamine, or prolamine-allied substance, not much hope of success in the production of solvents from hydrolysates of various natural materials was entertained. The addition of 5 per cent corn mash to the neutralized hydrolysates was found to provide enough prolamine to allow the fermentation to proceed normally. Conversion of the carbohydrate in the corn and in the hydrolysate was found to take place, giving a yield of solvents greater than could be obtained from five per cent corn mash alone. In view of the fact that hydrolysis of the various substances used in this work with hot dilute acid gave large yields of fermentable carbohydrate, and further acid hydrolysis of the residue yielded large additional quantities of sugar, this phase of the problem is worthy of further investigation. The only additional requirement which is apparently necessary for the fermentation of the hydrolysates is the presence of an alcohol-soluble protein or a substance closely allied to it. This can be supplied easily and cheaply in the form of yellow corn, which itself is capable of yielding large quantities of both acetone and butyl alcohol.

CONCLUSIONS

1. The strains of *Clostridium acetobutylicum* used in this investigation possess essentially the same morphological, cultural and physiological properties originally described for them by Weyer and Rettger (1927).

2. An alcohol-soluble protein or closely allied or associated sub-

stance appears to be necessary for the production of normal amounts of acetone and butyl alcohol from carbohydrate in a semi-synthetic medium.

3. Hydrogen-ion concentration, method of anaerobiosis, oils, concentration of peptone and type of medium used, had no apparent influence on the production of butyl alcohol from carbohydrate in a semi-synthetic medium to which a prolamine in some form had not been added.

4. In the absence of a prolamine or prolamine-allied substance the acidity curves for glucose, levulose, galactose, xylose, arabinose, sucrose, maltose, lactose, dextrin and starch do not agree with the curves derived by Robinson (1922).

5. In the presence of prolamine, or prolamine-allied substance, acetone and butyl alcohol are formed from xylose, arabinose and glucose in a ratio of 1:2. In the absence of the alcohol-soluble protein, normal amounts of acetone are produced, but little or no butyl alcohol.

6. The acid hydrolysis of the various raw materials used in this investigation gives good yields of reducing sugars. A second and more intense hydrolysis increases the total sugar yield materially.

7. Fermentation of the acid hydrolysates in the absence of a prolamine yields appreciable amounts of acetone only. The addition of yellow corn, which contains the alcohol-soluble protein, zein, stimulates the production of large quantities of both acetone and butyl alcohol.

8. Prolamine probably does not act as a catalyst in the reaction, or at least solely as a catalyst.

9. Butyl alcohol is not derived from the prolamine.

10. Alcohol-soluble proteins in all probability influence favorably the production of butyl alcohol from carbohydrate material in a semi-synthetic medium through a combination of their physical action and their direct influence on the metabolism of *Clostridium acetobutylicum*.

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EPIZOOTIC HEPATO-ENTERITIS IN GUINEA PIGS

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Received for publication, August 28, 1932

Late in 1931 and during the early months of 1932, a virus disease was first seen in a small guinea pig farm, near Guiza.

The following is a concise account of the disease.

SYMPTOMS

Incubation period on contact infection, seven to ten days and on subcutaneous inoculation with filtrates of body fluids, three to five days.

Rapid emaciation, loss in weight, cuddled appearance and a very rough coat were the very early signs, soon followed by profuse diarrhoea; faeces were not always seen tinged with blood, but loose and fairly offensive; exhaustion set in and the animals were seen lying on their side kicking and hopelessly attemptingto sit up and were usually found dead next morning!

POST-MORTEM

Chest cavity, occasionally lungs were seen congested, otherwise the respiratory organs looked apparently normal.

Abdominal cavity, liver congested, almost black in colour, gall bladder distended and full of bile which was not blood tinged. Spleen, kidneys and suprarenals slightly congested but not enlarged. Intestines inflamed. This was the prevalent P.M. picture and on rare occasions gastritis and peritonitis were also noticed.

BACTERIOLOGICAL FINDINGS

Blood and peritoneal exudates seeded anaerobically but no organisms were found; aerobically, occasional cocci were isolated but did not reproduce the disease.

A 1:40 blood saline Seitz filtrate (filter 1½ inches diameter, at room temperature, pressure 70 mm. for fifteen minutes, filtrate seeded with sterile result) in 0.5 cc. subcutaneous doses reproduced the disease with the before described picture; inoculated guinea pigs died on the fifth day of inoculation and filtrates of their blood reproduced the disease (10 guinea pigs used).

Rabbits, white rats, pigeons and fowls inoculated subcutaneously, intramuscularly or intravenously with filtered and non-filtered blood, diluted 1:40 with saline in up to 1 cc. doses and kept under observation for forty days did not suffer from the disease (6 animals of each species were used) while control guinea pigs (6 in number) died within the sixth day of subcutaneous inoculation with the same material used for other animals in 0.5 cc. doses).

Hence the virus is specific to guinea pigs.

IMMUNIZATION

Liver or spleen or both, mixed in paste emulsions and diluted with saline (1 cc. paste added to 40 cc. saline to which commercial formalin was added in 1:500 dilution) is protective in 1 to 2 cc. single doses,—25 animals were immunized, tested with 0.1 cc. 1:40 blood saline after fifteen days after the vaccine and proved immune, while controls, 5 guinea pigs, receiving the same test dose, died within the seventh day of inoculation.

Guinea pigs for research or diagnosis should be guarded since an outbreak of hepato-enteritis will certainly mask the diagnostic or research experiments carried out on guinea pigs.

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EXPERIMENTS WITH KENDALL'S MEDIUM[1]

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Received for publication, May 12, 1932

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Recently, Kendall (1931) has described a culture medium which is nearly free of altered protein and is peptone-poor. In this medium he was able to cultivate filterable cocci from the blood of influenza patients, individuals ill with the common cold, rabies virus and vaccine virus. Typhoid bacilli and streptococci were converted into filterable organisms in this medium and from staphylococcus bacteriophage, staphylococci were recovered. The apparent ease with which these transformations were effected has excited widespread curiosity and has led to this repetition of Kendall's work. Recently, Craig and Johns (1932) have attempted to repeat Kendall's observations without success.

METHODS

In every technical procedure Kendall's instructions were followed rigorously. The K medium was prepared as follows:

Fresh hog intestine was opened, cleaned and ground in a meat chopper. The ground material was immersed at once in four volumes of 95 per cent alcohol, and was extracted at 37°C. for two days with occasional stirring. The alcohol was then removed and fresh alcohol added. This was repeated twice, making three extractions in all. The dry tissue residue was then extracted with benzol and the benzol removed by filtration and then by an air current. The dried material was ground to a fine powder in a mortar and was kept in a stoppered container. The actual me-

[1] This investigation was supported by a grant from the John J. Abel Fund for Research on the Common Cold.

dium was prepared by adding 2 per cent by weight of the dried ground intestine to Tyrode solution. Small amounts of $NaHCO_3$ were then added and the final reaction was brought to pH 7.4. The medium was thoroughly mixed and tubed in 10-cc. amounts. Sterilization was accomplished by a single exposure to 15 pounds live steam pressure for twenty minutes in the autoclave.

The preparation of the test substances for cultivation in this medium was as follows: The various strains of typhoid bacilli were cultivated in K medium for forty-eight hours at 30°C., and after dilution with twice the volume of sterile physiological salt solution, were filtered through new Berkefeld V filters and inoculated into fresh K medium and control media. Staphylococcus bacteriophage was obtained by adding small amounts of bacteriophage to young growing cultures of *Staph. aureus* and permitting lysis to take place during an incubation period of eighteen hours at 37°C. The lysed cultures were filtered through new Berkefeld W and Seitz (Uhlenhuth model) filters. The filtrates were used to inoculate both the K medium and the control media. The filtrates of the N.Y.V. strain of the Beta hemolytic streptococcus were prepared in the same manner as the filtrates of the typhoid bacillus cultures. Rhinopharyngeal washings made with Tyrode's solution from individuals ill with early colds and filtered through Berkefeld W candles, were used as the source of the filterable agent of the common cold. A dermal strain of commercial vaccine virus was employed in the cultivation of vaccine virus.

Before inoculation, the sterile K medium was heated in boiling water to drive off the oxygen and then cooled rapidly in cold tap water. Inoculation was always carried out by adding to the K medium 0.5, 0.25 and 0.05 cc. of filtrate, respectively, to 10 cc. of the medium. The K medium cultures were incubated at 30°C. and the cultures were routinely examined for signs of growth at twenty-four, forty-eight and seventy-two hours, as well as at the end of seven and ten days. All cultures were preserved for a period of from three to six weeks before being finally discarded. Sub-cultures upon rabbits' blood agar were made at frequent intervals during the period of observation of the cultures and at the time when the cultures were discarded. Control tubes of

rabbits' blood broth and control rabbits' blood agar plates were inoculated from all filtrates and in every experiment uninoculated tubes of K media were included.

EXPERIMENTAL

Seventeen tests were carried out with three strains of *B. typhosus* (Rawlings, Hopkins and Goggle strains). All 17 tests were carried through one generation, while 10 experiments were carried through the second, and 6 through a third, generation. In no instance was there any evidence of the production of a "filterable" form of the typhoid bacillus with a subsequent transformation of this "filterable" form into a visible typical typhoid microörganism.

Twirty-two series of experiments were made with the Gratia strain of staphylococcus bacteriophage. Half of these tests were made with Seitz filtrates and the other half with Berkefeld W filtrates of the bacteriophage. In each instance 16 experiments were carried through one generation, 6 through two generations and 3 through the third generation of sub-cultures. All filtrates contained bacteriophage (as proved by their ability to bring about lysis in young growing cultures of staphylococci) when inoculated into tubes of K media. Staphylococci were not recovered from the K medium, the controls, or the rabbits' blood agar plate sub-cultures from the various generations of the K medium cultures. The four attempts to produce a filterable form of the N.Y.V. strain of Beta hemolytic streptococcus were likewise unsuccessful.

Five cultivation experiments, each carried through three generations, were made in an effort to recover filterable cocci from K medium inoculated with Berkefeld W filtrates of the rhinopharyngeal washings from individuals ill with common colds. These tests remained without results. Sixteen samples of a commercial strain of dermal vaccine virus were cultivated in the K medium. From 3 of the samples, diphtheroids, and from 3 others, *B. subtilis*, were cultivated in the first generation. Eight tests were carried through the second and third generations respectively without any signs of growth. It is well known that

vaccine virus occasionally contains non-pathogenic microörganisms and no importance was attached to the recovery of diphtheroids and *B. subtilis* from the vaccine virus.

CONCLUSION

Although Kendall's technique was followed minutely, filterable forms of *B. typhosus* and Beta hemolytic streptococci were not produced. It was not possible to cultivate staphylococci from staphylococcus bacteriophage, nor a filterable coccus from "cold" virus. Diphtheroids and *B. subtilis* were cultivated occasionally from commercial vaccine virus, but in view of the well-known observation that these organisms may be contaminants of vaccine virus, no importance was attached to these findings.

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THE DESICCATION AND PRESERVATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS[1]

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Received for publication, May 21, 1932

INTRODUCTION

During the investigation of infectious laryngotracheitis (Gibbs, 1931), the necessity of maintaining the virus in susceptible chicks proved to be of considerable importance, as much care and space had to be devoted to this part of the project, pending the discovery of a better method for preserving the virus. In a special communication, Beach suggested the Swift (1921) method, which he briefly reported later in connection with his own studies of infectious laryngotracheitis (1931). A perusal of the literature followed, in which it was found that Swift had preserved bacteria indefinitely in a viable state by simultaneously freezing and desiccating cultures of living microörganisms, and Stark and Herrington (1931) had kept bacteria for long periods of time by drying quickly at low temperatures without freezing.

The methods worked out by Swift and Stark and Herrington have been combined, modified, and adapted to the preservation of infectious laryngotracheitis virus in tracheal exudates. The object of this communication is to present the method found to be most satisfactory in our work.

In the preservation of infectious laryngotracheitis virus, freezing is unnecessary, although the exudates containing the virus should be kept at a low temperature, just above freezing, until completely dehydrated and protected from air, moisture, and direct sunlight. While the apparatus finally adopted as being

[1] Contribution number 141, Massachusetts Agricultural Experiment Station.

the most efficient in our hands for the desiccation of the virus is nearly as expensive as that used by Swift and Stark and Herrington, it is not as complicated.

APPARATUS

For the most efficient preservation of infectious laryngotracheitis virus, a large desiccator, 200 mm. inside diameter, should be provided. The Scheibler or the Frühling and Schultz types, when properly fitted with ground-in glass stopcocks, are satisfactory. Buchner tubes and smaller desiccators were tried, but they proved unsatisfactory because the air had to be evacuated more often in order to keep the moisture content down. The object in using a comparatively large desiccator is to provide space for the diffusion and dilution of the moisture as it comes out of the exudate and at the same time ample surface for an abundance of the dehydrating agent some distance away from the virus.

Most of the common dehydrating agents, such as anhydrous calcium chloride, acid phosphoric anhydride, and the commercial preparations, dehydrite and desicchlora, are satisfactory. The dehydrating agent should be selected for its ability to absorb large volumes of water and its rapidity of absorption.

The connections should be made with glass tubing as far as possible, and the limited amount of rubber tubing necessary for connecting the ends of the glass tubing to the pump, gauge, and desiccator should be designed especially for vacuum pumps. Castor oil will cause the rubber tubing to adhere to the glass connections more firmly than vaseline.

The sealing grease is important also. Vaseline is not satisfactory because of its low melting point, for when it is in the liquid state it is apt to permit the leaking of air into the deisccator from outside pressure, and after the desiccator has been placed in the icebox it tends to "freeze" the lid on. High melting grease as suggested by Van Slyke (1912) and Case (1932) is more satisfactory. The Van Slyke grease is made by dissolving one part of pure gum rubber, one part of paraffin, and two parts of vaseline

over a flame. The Case lubricant is made by the following procedure:

"To 100 cc. of medium grade lubricating oil add 2 grams of dry soap powder. Heat to 120 to 200°C. and stir for a few minutes or until the solution is complete. Add 20 grams of paraffin wax and stir well."

The addition of gum rubber to vaseline or of soap to lubricating oil forms a stiff jelly with a comparatively high melting point.

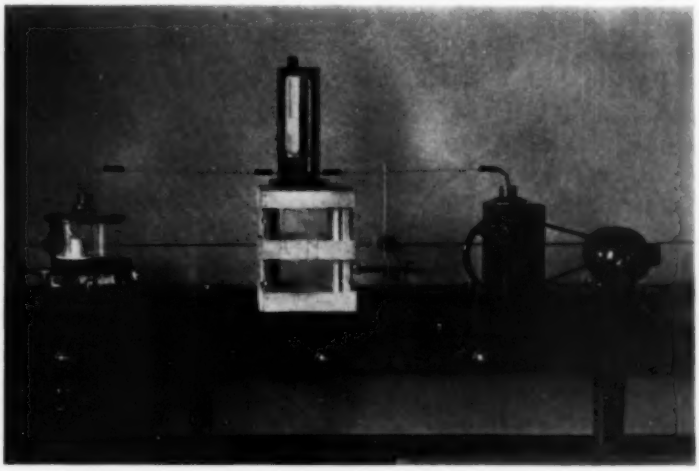

FIG. 1. DESICCATOR, MANOMETER AND VACUUM PUMP USED IN THE DESICCATION AND PRESERVATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS

The addition of paraffin wax to both forms a grease of uniform consistency, which permits a desiccator lid to slide easily even under considerable outside pressure.

METHOD

Before setting up the apparatus as shown in the illustration, the desiccator is heated to 150°C. in a drying oven for two hours or more, after which it is permitted to cool to 40°C. Then it is quickly removed from the drying oven, the anhydride and test

tubes containing the tracheal exudates placed inside, the cover and stopcock sealed with grease, and the air evacuated by means of the vacuum pump until the column of mercury is reduced to at least 300 microns, as measured by a McLeod gauge.

A manometer may be substituted for a McLeod gauge, as shown in the illustration, provided it has been previously tested by a more sensitive instrument and comparative readings taken at different barometric pressures for the guidance of the operator.

As soon as the vacuum inside of the desiccator has reached a satisfactory point, as indicated by the gauge or manometer, the stopcock is closed and the desiccator is disconnected from the system and rotated carefully so as to permit the anhydride to come in contact with every part of the inside wall. For the most uniform results, the desiccator should be evacuated once a day and rotated each time before it is returned to the ice box. The operation should be repeated four or five days in succession, or until the vacuum remains constant without the aid of a pump.

After standing in the ice box a day or two longer, the desiccator may be opened, the tubes removed, the cotton plugs pushed down an inch, and the open space promptly filled with melted paraffin or sealing wax, after which the tubes are returned to the icebox. When it is desired to use the virus the test tubes should be removed from the icebox, the paraffin or wax melted and the cotton plugs pulled out. The dried tracheal exudates should then readily slide out of the tubes without adhering to the sides.

THE VIRULENCE OF DESICCATED INFECTIOUS LARYNGOTRACHEITIS TRACHEAL EXUDATES

Table 1 shows the results of the desiccation and preservation of tracheal exudates of infectious laryngotracheitis, by days, in a large 200 mm. desiccator evacuated by a vacuum pump, some of them to less than 300 microns of mercury, and the others to 300 microns or more of mercury. Acid phosphoric anhydride was used as the dehydrating agent. Three strains, designated as the California, New Jersey, and Massachusetts viruses, were selected for this study. These viruses had been used in other experiments for nearly three years and were fairly representative of infectious

laryngotracheitis viruses in general. The virulence tests were made by intratracheal inoculations in chickens four weeks old which had never been exposed to infectious laryngotracheitis. The inoculated chickens were isolated in individual cages and kept under observation for symptoms of the disease for three weeks.

In a vacuum of less than 300 microns ten of the California viruses were virulent and five avirulent. This order was reversed

TABLE 1

DAYS PRESERVED	VACUUM FOR TRACHEAL EXUDATES					
	Less than 300 microns			300 microns or more		
	California	New Jersey	Massachusetts	California	New Jersey	Massachusetts
30	0	+	0	+	+	+
60	+	0	0	+	+	+
90	+	0	+	+	+	+
120	+	+	+	+	+	+
150	0	+	+	+	+	+
180	+	0	+	+	+	+
210	+	0	+	+	+	+
240	+	0	0	+	+	+
270	+	+	+	+	+	+
300	+	0	+	+	+	+
330	+	0	0	+	+	+
365	+	+	+	+	+	+
390	0	0	0	+	+	+
420	0	0	0	+	+	+
450	0	0	0	+	+	+

+, virulent; 0, avirulent.

for the New Jersey virus in which five of the desiccated tracheal exudates were virulent and ten avirulent. The Massachusetts virus occupied an intermediate position in that eight were virulent and seven avirulent. None of the viruses were preserved for periods longer than 365 days, or one year. This part of the experiment indicates that a vacuum of less than 300 microns of mercury, as measured by a McLeod gauge, is not sufficient for desiccating and preserving infectious laryngotracheitis virus regularly over long periods of time.

In the other part of the experiment, however, in which a vacuum of 300 microns or more of mercury, as measured by a McLeod gauge, was used in the process of desiccation, all three of the viruses were found to be preserved at regular monthly intervals up to 450 days without any apparent attenuation in virulence.

DISCUSSION

The preservation of infectious laryngotracheitis virus in tracheal exudates by drying presents some problems of theoretical interest, for the facts appear to be contrary to the accepted view that life in the complete absence of water is impossible. Furthermore, the virus occurs naturally in the larynges and tracheas of susceptible birds, a location abundantly supplied with oxygen, yet after the birds are dead or the virus is removed from its natural environment both combined and free oxygen seem to be unnecessary, if not detrimental to its existence. As pointed out by Stark in an analogous case with bacteria, there are three types of waters concerned in the desiccation of tracheal exudates,—free water, bound water, and water of constitution. During the process of drying, some of the bound water is set free and all of the free water is removed, either in the air, evacuated from the desiccator or absorbed by the dehydrating agent. But as yet it is impossible to determine the proportion of bound water set free or whether any of the water of constitution is removed from the exudates or not. As to the part played by oxygen, it may be reasoned that oxygen is combined in the tissues and that during the process of desiccation the combined and free oxygen of the air only is removed, thus preventing any further oxidation and enzymic action. Apparently, the desiccation of tracheal exudate *in vacuo* preserves the virus of infectious laryngotracheitis indefinitely because the moisture necessary for bacterial decomposition and the oxygen essential for oxidation and enzymic action are removed. But in order to be effective the removal of these substances should be done in a thoroughly scientific manner with the proper equipment, for speed and thoroughness of removal of air and moisture seem to be the most important factors.

SUMMARY

A laboratory method is described for the desiccation and preservation of infectious laryngotracheitis virus.

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A WEIGHTED MEAN METHOD OF AVERAGING BACTERIAL COUNTS OF MILK

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Received for publication, June 10, 1932

In the JOURNAL OF BACTERIOLOGY for February, 1932, A. H. Robertson presents a method by which a single figure may be obtained to represent the average of a series of observations, such as the bacterial counts of milk, where premiums to be paid to dairymen are assessed by such counts.

All bacteriologists who have studied such a problem realise the difficulties involved in attempting to condense the results of a long series of counts into one figure and, if they have consulted an orthodox mathematician, have probably been met with a *non possumus* attitude, engendered by data so scattered as to give a standard deviation twice as great as the mean.

While, mathematically, it may be quite illegitimate to deduce a single figure from such data, it is, in many cases, a practical necessity to do so, and it would be a matter of considerable utility if common agreement were obtained as to a method.

Robertson has suggested the mathematically improper method of averaging logarithms. He defends this procedure by emphasizing the logarithmic phase of the rate of growth of bacteria. But where we are dealing with different samples of milk, kept under different temperature conditions and possibly for somewhat different times, it seems doubtful if this defence is a good one. We would, however, be willing to adopt Robertson's method if it gave useful results even if the theoretical basis on which it is founded were weak.

Robertson, in his paper, gives a series of 174 counts, obtained from samples of one dairyman's night milk over a period of

nearly four years. As Robertson points out, the mean is 20,500. Only 44 counts exceed the mean, while 130 are below it. In this case the mean is elevated to an unreasonable extent by four counts above one million.

What we wish to assess is really the degree of care exercised by this particular dairyman in the production of milk. If we take counts of not more than 10,000 per cubic centimeter as the result of extreme care, counts of 10,000 to 30,000 great care, counts of 30,000 to 100,000 moderate care and counts over 100,000 as evidence of carelessness, this dairyman, in 174 tests, was extremely careful 93 times, very careful 50 times, moderately careful 27 times and careless only 4 times. It is just that he should suffer financially for the 4 occasions on which he was careless, but it is no less just that he should be rewarded for the 143 occasions on which he was extremely or very careful. His reward should certainly be less than that of a competitor who, in 174 tests, was never found guilty of carelessness, but the disparity in the awards of the two should not be very great.

Some twelve years ago, the senior author had to deal with a similar problem and a method was evolved which, it is believed, is superior to that of Robertson. It is based on the inclusion of all the figures, but much greater weight is attached to the median and the counts in close proximity to it than to the exceptionally high or low counts.

The method is best explained by taking the series given in the first column of table 4, p. 130 of Robertson's paper. Details of the work are shown in table 1.

1. The figures are arranged in order of magnitude from greatest to least.

2. They are written in two columns A and B. In the first line of A is the highest figure, in the first line of B the lowest. In the second line of A is the next highest figure and in the second line of B the next lowest. This process is continued until all the figures are exhausted. If the series contains an even number of items the two central of these appear in the last lines of columns A and B. If the series is odd, the median is entered in the last line of A.

3. In the first line of C is written the sum of all the figures in columns A and B; in the second line this sum, less the figures in the first lines of A and B; in the third line the sum, less the figures in the first and second lines of A and B and so on until, in the last line of C, we write the sum of the two median items if the series is even, or the median if the series is odd.

TABLE 1

A	B	C	D	E
346,500	1,600	999,500	44	22,715
60,900	2,200	651,400	42	15,509
55,300	2,300	588,300	40	14,707
51,800	2,300	530,700	38	13,965
41,300	2,600	476,600	36	13,238
38,500	3,000	432,700	34	12,726
36,400	3,000	391,200	32	12,225
29,400	3,500	351,800	30	11,726
25,900	3,600	318,900	28	11,389
25,900	5,100	289,400	26	11,130
17,700	6,500	258,400	24	10,766
16,100	6,700	234,200	22	10,563
16,000	6,900	211,400	20	10,570
14,900	7,100	188,500	18	10,472
14,900	7,100	166,500	16	10,406
14,000	7,300	144,500	14	10,321
13,300	7,800	123,200	12	10,266
12,000	8,500	102,100	10	10,210
11,500	8,700	81,600	8	10,200
11,500	8,900	61,400	6	10,233
11,400	9,100	41,000	4	10,250
10,800	9,700	20,500	2	10,250

Sum of items in column E	263,837
Number of items in column E	22
Weighted mean	12,000

4. In each line of column D is entered the number of items summed in the corresponding line of C.

5. The figures in column E are obtained by dividing the corresponding figure in column C by that in column D. The first figure in E is therefore the arithmetic mean of the series, the second the mean of the series with the highest and lowest items

omitted, the third the mean of the series with the two highest and two lowest omitted and so on. The last figure is the median or the mean of the two central items.

6. The figures in column E are added and divided by their number. The result is the weighted mean which is taken as representative of the whole series. It has been weighted by paying most attention to the figures centering round the median.

For this short series the following means have been obtained:

Arithmetic mean	22,700
Logarithmic mean	10,800
Weighted mean	12,000

While there can be no doubt that the arithmetic mean is too severe on a comparatively few bad results it is believed that the logarithmic mean is rather lenient and it is suggested that the weighted mean gives a more truly representative result.

For the whole series of results given by Robertson the following are the various means:

Arithmetic mean	20,500
Logarithmic mean	10,200
Weighted mean	11,200

Here again it seems to us that the weighted mean more nearly represents the average degree of care exercised by the producer of the milks than does the logarithmic mean.

The best way of testing the two methods is by comparing the four theoretical series of counts W, X, Y and Z shown in table 2. In each case 19 counts are given. The standard figure, denoting the exercise of proper care, is taken as 10,000. In series W the proper degree of care, neither more nor less, was displayed all through. In series X, 10 samples show the standard degree of care but in the other 9 samples greater care has produced milks of increasing bacterial purity. Series Y contrasts with series X in that, while the standard figure is maintained in 10 samples, the remaining 9 samples exceed the standard each to the same extent as did the 9 in series X fall below it. In series Z there is a lack of uniformity which is regular, so that for each milk giving a count above the standard there is another giving a count an equal

amount below the standard. For series X, Y and Z the percentage differences of the weighted and logarithmic means above or below the 10,000 standard are given.

Let us suppose that the producer of milk giving a mean count of 10,000 is to receive a basic rate of pay, that a producer whose

TABLE 2

	W	X	Y	Z
	10,000	10,000	19,000	19,000
	10,000	10,000	18,000	18,000
	10,000	10,000	17,000	17,000
	10,000	10,000	16,000	16,000
	10,000	10,000	15,000	15,000
	10,000	10,000	14,000	14,000
	10,000	10,000	13,000	13,000
	10,000	10,000	12,000	12,000
	10,000	10,000	11,000	11,000
	10,000	10,000	10,000	10,000
	10,000	9,000	10,000	9,000
	10,000	8,000	10,000	8,000
	10,000	7,000	10,000	7,000
	10,000	6,000	10,000	6,000
	10,000	5,000	10,000	5,000
	10,000	4,000	10,000	4,000
	10,000	3,000	10,000	3,000
	10,000	2,000	10,000	2,000
	10,000	1,000	10,000	1,000
Weighted mean	10,000	8,800	11,200	10,000
Logarithmic mean	10,000	6,600	12,000	7,800
Percentage difference above or below 10,000:				
Weighted mean, per cent		−12	+12	0
Logarithmic mean, per cent		−34	+20	−22

milk exceeds this number is to be penalised by a proportionate reduction in pay and that the producer whose milk gives a lower count is to be rewarded by a proportionate increase in pay.

Under this scheme, when the weighted mean method is employed X will receive a bonus of 12 per cent, Y will suffer a deduction from the basic pay of 12 per cent and Z will get exactly the

basic pay. Mathematically it seems fair that X's bonus should equal Y's penalty, since the latter exceeds the standard by as much as the former falls below it and also that Z should receive the basic pay since each milk worse than the standard is balanced by one an equal amount better.

When calculations of pay are made by the logarithmic mean method, the unfairness of this method is at once apparent. X receives a bonus of 34 per cent and from Y is exacted a penalty of 20 per cent. By our method the reward and the penalty were equal, as from mathematical reasoning they should be. While, in the case of milk, it might be better to penalise Y to a greater extent than we reward X, since increases of bacteria above the standard are more prejudicial than decreases below this standard are beneficial, there seems to be no justification for making X's reward greater than Y's penalty.

An examination of Z's results is still more striking. As judged by the logarithmic mean method, Z should receive a premium of 22 per cent as compared with W, although any excess of care displayed by him is exactly balanced by an equal display of carelessness. By our method Z should receive the basic rate of pay. Actually it might be better if he were slightly penalised, but it seems quite wrong that he should be rewarded as he would be if his score was calculated by the logarithmic mean method.

Since it is obvious that the weighted mean method is inapplicable in the case of a series of two items, it might be thought that it would give fallacious results if applied to any small series, such as one consisting of three or four items. If this were so it would be impossible to recommend the method for general use, as milk companies are commonly obliged to assess payment to milk producers on quite small series of counts. It cannot, however, be over emphasised that the smaller the series, the less reliable are the results of averaging counts by any method. In our opinion no attempt should be made to average a series of less than four items.

In order to test the two methods further, theoretical series of counts L, M, N and O, each of four samples and P, Q, R and S, each of three samples were constructed in the same way as

were series W, X, Y and Z. These, together with the means, are given in tables 3 and 4.

An examination of these figures will at once show that the criticisms of the logarithmic mean method made on the results of the long series given in table 2 apply with equal or even greater

TABLE 3

	L	M	N	O
	10,000	10,000	19,000	19,000
	10,000	7,000	16,000	11,000
	10,000	4,000	13,000	9,000
	10,000	1,000	10,000	1,000
Weighted mean	10,000	5,500	14,500	10,000
Logarithmic mean	10,000	6,500	14,100	6,600
Percentage difference above or below 10,000:				
Weighted mean, per cent		−45	+45	0
Logarithmic mean, per cent		−35	+41	−34

TABLE 4

	P	Q	R	S
	10,000	10,000	19,000	19,000
	10,000	5,000	15,000	10,000
	10,000	1,000	10,000	1,000
Weighted mean	10,000	5,200	14,800	10,000
Logarithmic mean	10,000	3,700	14,200	5,700
Percentage difference above or below 10,000:				
Weighted mean, per cent		−48	+48	0
Logarithmic mean, per cent		−63	+42	−43

force when the series is small. It is particularly important to note that, if we rely on the logarithmic method, O and S, who exercised less care than the basic amount (as denoted by a count of 10,000) as frequently and to the same extent as they exercised more care, would be rewarded to the extent of 34 per cent

and 43 per cent respectively as compared with L and P. It seems to us quite unfair that O and S should be rewarded; we would prefer, if the method permitted it, that they should be penalised.

Comparing the percentage differences above or below 10,000 given in tables 2, 3 and 4, one is tempted to deduce that the results obtained by the logarithmic mean method become more and more illogical as the number of items in the series is reduced, while the weighted mean method, applied to any series of three or more items, gives consistently good results.

We contend that the weighted mean method which we advocate for the scoring of bacterial counts in milk is impartial and equitable and that it is preferable to Robertson's logarithmic method as it gives results which more nearly represent the average quality of the milk supplied. This method has another advantage—the figure on which payment is calculated is deduced by simple arithmetical operations. Logarithms are an unknown quantity to most milk producers and their use in assessing a reward, or still more a penalty, is liable to be regarded with the gravest suspicion.

SUMMARY

1. A weighted mean method of estimating the average bacterial count of a series of individual counts of milk is described.

2. The weighted mean is calculated by finding the mean of a series of means: (a) of the whole series; (b) to the whole series less the highest and lowest; (c) of the whole series less the two highest and two lowest and so on.

3. It is claimed that the weighted mean impartially and equitably reflects the average bacterial content of the milks and that it is a fairer method than the logarithmic mean method described by Robertson.

4. The method is applicable to any series of three or more items but, as with any method, the larger the number of items the greater is the reliability of the result.

CYTOLOGICAL CHANGES DURING THE FORMATION OF THE ENDOSPORE IN BACILLUS MEGATHERIUM[1]

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Reviewers of our knowledge of the mode of formation of endospores in bacilli usually present the evidence under one or more of three theses: (1) the endospore is formed by the growth of a specialized granule in the bacterial protoplasm; (2) the endospore is formed by the fusion of a number of granules, some of which may be nuclear material; (3) the endospore is formed by gradual condensation of cell-substance, possibly due to dehydration. These notions have been derived from the study of both stained and unstained bacilli. Although the origins of these conceptions are obscure, they can be associated with names of distinguished bacteriologists. The first of these opinions was expressed by Koch (1876) in his description of the growth of the spore from a granule in *Bacillus anthracis*. de Bary (1887) noted the growth of the spore from a granule in *Bacillus megatherium* and Ward (1895) described the same process in *Bacillus ramosus*. Ernst (1888–1889) and Babes (1889) are the sponsors of the second theory of spore-formation and their view of the origin of the spore in the fusion of granules, composed chiefly of nuclear material, has been corrected and extended in modified form by Schaudinn (1902) and Dobell (1908, 1909, 1911). The third conception of spore-formation by protoplasmic condensation has been in the minds of many investigators. Matzuschita (1902) mentioned it definitely and lately von Darányi (1927, 1930) has emphasized it as a consequence of his observations on the effects of col-

[1] Presented at the thirty-third annual meeting of the Society of American Bacteriologists in Baltimore, Maryland, December 28, 1931.

loidal dehydration in bacteria. Probably most bacteriologists accept the opinion of Koch and de Bary and would agree with Cook (1932), who in his recent paper on bacterial spores, states that de Bary's account of the growth of the spore from a granule could not be bettered. A review of the original reports of observations on unstained bacilli, however, arouses some doubt as to whether the methods of periodic visual observations and staining procedures used in those studies were actually capable of yielding sufficient information to support the conclusions drawn from them. Furthermore, any attempt to watch the random behavior of granules in bacilli during spore-formation will excite both bewilderment and skepticism in the observer. An additional basis for doubting the opinion that the spore grows from a granule or granules is furnished by the recent work of Wyckoff and Ter Louw (1931), who have reported that even in photographs made with ultraviolet light they were unable to detect a precedent granule in the sporogenous area in *Bacillus subtilis*. As the observations of Wyckoff and Ter Louw were not continuous from the commencement to the end of spore-formation, their work cannot be said to settle the question. While it is not beyond the range of possibility that all these three modes of endospore-formation may occur in a single or different species of bacilli, it seems probable that so morphologically definite a phase in the life-cycle of some bacteria should occur according to a uniform process. At present, however, the force of the published observations does not compel the acceptance of any one of the three prevalent conceptions of the process of endospore-formation.

The formation of a spore in a bacillus is a cellular process composed of both slow and rapid phases. Neither can be followed by the eye. Continuous visual inspection of a slow cellular process over long periods is impossible on account of fatigue of the eye. Periodic records of visual impressions are uncertain and may lead to false conclusions because of unobserved changes taking place in the intervals between observations. Periodic photography overcomes many of these difficulties, as far as the optical resolution of objects and the registration of images will permit. This method becomes especially useful when the intervals between

the photographs are shortened by means of motion picture apparatus. The record upon the film, when projected rapidly, presents a dynamic reproduction of the process occurring in a selected cell or cells. When used for the study of individual frames, projected at convenient magnification as single pictures upon a screen, this record gives material suitable for analysis by measurement and by prolonged and repeated study of details. Time intervals between stages can be accurately determined from the film. This kind of record presents, finally, the consecutive changes occurring in an unstained microorganism, undisturbed by manipulations, and growing under as natural conditions as an optically serviceable culture chamber will permit. Whenever motion photomicrography can be applied to the investigation of a process or cyclical stage in a minute organism, it would seem to us to be the method of choice.

For these reasons, since 1928 we have been investigating the process of endospore-formation in *Bacillus megatherium* by the method of motion photomicrography. We have not investigated the cytological changes by means of staining reactions. The description and analysis of the record obtained upon the films, to be presented in this paper, confirm the observations of some observers, are definitely opposed to the opinions of others and seem to us to add somewhat to the knowledge of the cytological changes occurring during this process.

METHODS AND MATERIALS

The apparatus for motion photomicrography used by us in these studies was essentially the same as that devised by Bayne-Jones and Tuttle (1927). It has been modified by driving the camera by means of a synchronous motor through a set of adjustable accurate gears and by the insertion of a pentaprism in the view finder. Exposures were made at the rate of 7 per minute. The film used was 16 mm. panchromatic reversal film. The source of light was a tungsten ribbon filament 6 volt, 108 watt lamp, set up 1 meter from the mirror of the microscope and adjusted to give critical illumination. Between the lamp and the mirror were interposed a B_{58} green Wratten filter (transmission

wavelengths 460–600mμ), neutral density filters from 0.6 to 0.9, and a glass cell 5.5 cm. thick containing a saturated aqueous solution of quinine bisulfate to absorb heat and some of the shorter wavelengths of light. The intensity of the light falling upon the preparation was still further reduced by the substage diaphragm of the microscope to a degree just suitable for sharp visual observation through the view finder. The microscope objectives used were either a 1.8 mm. fluorite objective (N.A. 1.3) or a 3 mm. apochromatic objective (N.A. 1.4). The 1.8 mm. fluorite objective proved to be the more serviceable. With a ×3 negative ocular, this gave a magnification of ×300 on the film, with sufficient resolution and sharpness of focus to allow the use of a 25 fold enlargement of single frames for measurements.

The microscope was enclosed in an incubator with temperature regulation at 37° ±0.1°C.

The shutter of the Model A Ciné-Kodak which we used requires that the illumination be continuous. This would have its disadvantages if it were necessary to use bright illumination for a long time, since we have seen that the growth of yeast and bacteria is inhibited by intense light in this spectral region. But long experience with our apparatus showed us the limits of the effects of our illuminant and we have never seen any deleterious action produced by the relatively dim light focussed upon the preparations photographed for these studies.

The culture chamber consisted of a cell 0.5 mm. deep. It was made by cementing a glass ring, 17 mm. in diameter, upon a clear microscope slide 1 mm. thick. A mixture of beeswax, paraffin and rosin was used as the cement, as this remains hard at 37°C. The slide, ring and cover glass were sterilized in a flame. A thin layer of the melted wax was placed upon the bottom and top borders of the 0.5 mm. ring. The ring was then attached to the slide. In the center of the ring a small cone of agar-peptone medium was built up by placing, with a sterile capillary pipette, drops of molten agar in this position until the top of the cone reached the upper level of the ring. When the agar hardened, the top of the agar cone was inoculated with bacteria by means of a fine platinum needle. A No. 1 cover glass was then laid over this, pressed down

and cemented to the ring. Usually a small space for the passage of air was left open on one side between the under surface of the cover glass and the top edge of the ring. When the cover glass was cemented in place, the top of the agar cone was flattened, giving a circle 3 to 4 mm. in diameter. In this area, the bacteria lay in a shallow layer, usually the depth of the thickness of a single organism. They grew in this layer, apparently without compression or obstruction, and spread over the flat surface of the medium in a single layer. Hanging drops of agar were tried at times, but their optical properties were not as satisfactory as those of the shallow agar cone and they tended to dry too quickly.

Detailed descriptions of the apparatus, culture method and method used in measuring the images on the films are given by Bayne-Jones and Adolph (1932).

We have used several cultures of *Bacillus megatherium* during the course of our studies. The strain employed for the observations reported here was a Kral Collection culture of *Bacillus megatherium*, sent to us by Dr. P. B. Cowles in August, 1930. Its morphological and cultural characteristics were essentially the same as those listed for *Bacillus megatherium*, de Bary, on page 396 in the 1930 edition of Bergey's Manual of Determinative Bacteriology, except that its endospores were formed nearer the terminal portions of the rods than in the central area. In germinating, the spores became round, then elongated and opened at one end, showing a type of polar germination.

FACTORS INFLUENCING SPORE-FORMATION

The conditions influencing the production of endospores by *Bacillus megatherium* were discovered to be very precise. It was necessary in the culture cell, to provide for an apparently optimal balance of oxygen tension, moisture and nutriment. We have accumulated a considerable amount of information upon the factors influencing spore-formation in this and other species of bacteria. While we shall present here only those details which bear directly upon spore-formation under the conditions imposed by our photographic arrangements, we know from experiments with larger cultures in test-tubes that factors influencing spore-forma-

tion in a layer of growth upon an agar cone below a coverslip are in general the same as those affecting spore-formation under other conditions of cultivation. With *Bacillus megatherium*, it seemed to us that an adequate supply of oxygen in proportion to the amount of growth was more important than the exhaustion of the medium. Wund (1906) showed that *Bacillus megatherium* failed to produce spores on an adequate medium when the oxygen concentration of the gas over the culture was reduced below 1.13 per cent. It is probable, also, that loss of water from the medium tends to induce spore-formation at a stage of growth when an excess of moisture might inhibit or delay spore-formation.

It was always necessary to provide an initially adequate medium for the growth of the vegetative cells, and we noted, in general, that an unfavorable environment inhibited spore-formation.

We have seen appearances in the cells of *Bacillus megatherium* indicating that endospore-formation has a sort of momentum. Cells from fourteen- to sixteen-hour cultures, containing cleared areas, which we have learned to recognize as the sites of spore-formation, proceed to complete endospore development when transferred from old to fresh medium. In such instances, spore-formation, once initiated, continued to completion, although the cell was removed from waste products and provided with a new supply of food-stuffs. When these cells in the early stages of spore-formation failed to complete the process on fresh medium, they remained in that stage. We have never seen a cell in the preliminary stage of spore-formation reverse the process and resume its vegetative multiplication.

Since the bacterial spore serves to perpetuate the organism through hardships, it has been assumed that the spore develops in response to unfavorable environmental conditions. Our observations on the factors influencing the formation of the spore are opposed to this view. Conditions close to optimum conditions for multiplication must be provided in order for the organism to progress to the stage of spore-formation. We agree with Cook (1932) that the teleological argument does not give a true indication of the primary function of the spore.

CONDITIONS PROVIDED FOR GROWTH AND SPORE-FORMATION

When planted upon a cone of medium composed of 2 per cent agar, 2 per cent peptone, 0.5 per cent NaCl, with a pH of 6.8 to 7.4., *Bacillus megatherium* grew abundantly under the cover slip but few or none of the cells developed endospores. We assumed that the organism used oxygen faster than it could diffuse into the growth, preventing the maintenance of an oxygen tension suitable for spore-formation. On the same agar medium in a thin hanging drop in the culture cell, spore-formation occurred in fourteen to sixteen hours in the cells growing between the cover glass and the agar. In this case it seemed that there was an adequate diffusion of oxygen through the thin layer of medium. This type of preparation, however, was optically poor and subject to excessive drying. Finally, we found that reduction of the peptone content of the agar medium to 0.05 per cent gave dependable preparations. Growth on the 0.05 per cent peptone, 2 per cent agar cone, beneath the cover glass proceeded in a normal manner but came to an end before the available area of the medium was too densely crowded with bacteria. This might be called a "starvation" medium. Nevertheless, it did not seem to cause abnormalities in the culture. In the early stages of growth the cells were large and hyaline, similar to those occurring on a medium composed of 2 per cent peptone and 2 per cent agar. In the late stages, the cells were the same size as, or slightly smaller than, cells measured by Adolph and Bayne-Jones (1932) from motion photomicrographs of the growth on 2 per cent peptone agar. The effect of the reduced peptone content was to shorten the period of vegetative multiplication so that the cells reached the age appropriate for spore-formation at a time when the environmental conditions were still favorable for spore-formation. Growth almost reached its end in 16 to 19 hours, although some cells, which were not producing spores, continued to enlarge twenty-one hours after inoculation. We assumed that the retardation of growth on 0.05 per cent peptone agar in the culture cell occurred before all the available oxygen was used or that the diffusion of oxygen could keep pace with the less abundant growth, permitting the main-

tenance of an oxygen tension suitable for spore-formation. The outcome of the experiments accorded with this prediction, but did not, of course, prove the correctness of the assumption. Undoubtedly, the conditions are complex. We did not make the quantitative measurements of each factor which would be needed to evaluate its separate influence.

OBSERVATION ON CYTOLOGICAL CHANGES

The cellular changes occurring during the final stages of spore-formation are shown in the motion picture frames reproduced in plate 1, figures 1, 2, 3 and 4. A description of the process is as follows:

After the inoculation of the 0.05 per cent Difco peptone, 2 per cent agar cone with *Bacillus megatherium*, the organisms under the cover slip do not change during the next thirty to ninety minutes. After this period of lag, growth proceeds rapidly. The cells are at first large and hyaline. Fine refractile granules appear in the cells about 3 hours later. Occasionally one or more large refractile granules appear at this time. As the culture becomes older, round granules of various sizes become very numerous in the cells. These granules are in rapid random motion. Some of the granules dart rapidly from one end of the cell to the other. There is no evidence of a streaming of granules to indicate a flowing of the protoplasm. In some cells, granules half as large as a mature spore appear and become surrounded by a clear zone. Two of these large granules may appear in a single cell. They have no connection with the formation of the spore, since they remain as granules in the cell after the development of the spore. The activity of the movement of the granules and their rapid transit at times from end to end of a cell seem to indicate that the protoplasm of the cell at this stage is quite fluid, with low viscosity. When the cells become old, or die or begin to disintegrate after spore-formation, the movement of these granules ceases.

From the eighth to the thirteenth hour after inoculation, when multiplication has stopped in most of the cells and when growth is continuing only slowly in others, the ends of the rods begin to

lose their granules. It is difficult to count and trace the granules in these cells because moving granules drop out of focus, and new granules may be formed. It is our opinion, however, that the ends of the cells become clear of granules by a displacement of the granules from that region and not by a liquefaction or fusion of the granules previously visible there, or by the growth of a special granule in this area. The clear area at the end of the cell fluctuates in size and gradually comes to occupy a cylindrical volume equal to approximately one-quarter of the volume of the cell. The material in this cleared area appears to be denser than the rest of the protoplasm of the cell. Granules in violent motion in the remainder of the cell strike against the edge of this cleared area but never penetrate it. This large cleared area is the region in which the spore appears.

The clear area at the end of the cell keeps a roughly cylindrical form for three to four hours. Its density increases gradually and it gradually assumes an almost spherical shape (plate 1, fig. 1).

The completion of the formation of the endospore, after the cleared dense area of protoplasm begins to take on a definite form, occurs with astonishing rapidity. The rest of the process is finished within the next thirty minutes. A stage which we have called the "prespore" appears as a dense refractile region in this cleared area at the end of the cell. Its volume is smaller than that of the original clear area but often twice that of the final spore. Within six to twelve minutes, this nearly spherical "prespore" contracts to an elongated ellipsoid and has a density, as judged by its refractile property, as great as that of the mature spore. This is shown in cell A in plate 1, figure 3. The optical section of the focus used makes these bodies appear black. Neither they nor the spores were stained in these preparations. Within six minutes or less, this elongated ellipsoidal spore contracts to the somewhat shorter, but apparently no broader ellipsoidal spore, characteristic of this organism.

During these terminal stages of spore-formation and after the development of the spore, the movement of the granules becomes less and less in the remaining protoplasm of the cell. Nevertheless, some of the granules may now pass into the area near the end

of the cell, along the side of the spore, into a region that was too dense to admit them before the contraction of the sporogenous area.

We have not seen the development of a membrane around the material of the spore.

Measurements of the dimensions and volumes of 12 cells with contained endospores are presented in table 1. The time intervals of the visible stages of spore-formation are shown for cells A to F. The measurements were made by projecting the single frames of the film on a screen at an enlargement of 25 times, taking the lengths and breadths of cells and spores with calipers and measuring these distances on a millimeter rule. The magnification of the images of the cells on the film was ×300. This 25 fold enlargement gave a magnification of ×7500. The pictures were sharp enough to be measured with an error within 10 per cent. We think that these measurements reduced to micra are accurate to within ±0.1 micron. With a film of pictures free from distortion due to shifts in the focus of the microscope, we have obtained more consistent and apparently more accurate measurements of bacteria than we have by the use of a filar micrometer or ocular micrometer.

Since the spore of *Bacillus megatherium* does not swell its sporangium, no local enlargement of the cell was to be expected. The measurements and calculations show that there was little or no increase in length or breadth of the cell and no significant change in volume while the spore was developing.

The volume of the cell was calculated as for a cylinder, using the formula: volume $= \pi/4 \times$ length $\times$ square of breadth. The spores were regarded as an ellipsoid of rotation around the long axis, with volume $= \pi/6 \times$ length $\times$ square of breath.

The volumes of the cells containing endospores varied from 6.9 to 12.5 cubic micra. The volumes of the contained spores were 0.55 to 0.97 cubic micron, with an average of 0.84 cubic micron. The average ratio of spore-volume to cell volume was 0.09. But this average obscures some considerable differences in this ratio, which varied from 0.06 to 0.13. Spores of the average size were formed in both large and small cells.

TABLE 1

Dimensions and volumes of cells and contained endospores at different intervals during spore-formation

CELL NUMBER	TIME AFTER INOCULATION		CELL			SPORE			STAGE
			Length	Breadth	Volume	Length	Breadth	Volume	
	hours	*minutes*	μ	μ	*cu* μ	μ	μ	*cu* μ	
A Fig. 1	14	0	4.6	1.5	8.1	1.6	1.3	1.41	Prespore
		6	4.6	1.7	10.3	1.5	1.2	1.13	Prespore
Fig. 2		12	4.6	1.7	10.3	1.7	1.2	1.28	Prespore
		18	4.6	1.7	10.3	1.6	1.1	1.03	Prespore
Fig. 3		24	4.6	1.7	10.3	1.5	0.9	0.64	Spore
Fig. 4		30	4.6	1.7	10.3	1.5	0.9	0.64	Spore
		36	4.6	1.6	9.3	1.6	0.9	0.68	Spore
B Fig. 1	14	0	3.5	1.5	6.2	1.5	1.1		Clear area
		6	3.6	1.5	6.3	1.5	1.1	0.97	Prespore
Fig. 2		12	3.6	1.5	6.3	1.5	1.2	1.13	Prespore
		18	3.5	1.5	6.2	1.5	1.1	0.97	Prespore
Fig. 3		24	3.5	1.5	6.2	1.5	1.1	0.97	Prespore
Fig. 4		30	3.5	1.5	6.2	1.5	1.1	0.97	Prespore
		36	3.5	1.5	6.2	1.5	1.2	1.13	Prepsore
C Fig. 1	14	0	5.3	1.6	10.6	1.7	1.3		Clear area
		6	5.5	1.7	12.5	1.6	1.3		Clear area
Fig. 2		12	5.3	1.7	12.0	1.3	1.3		Clear area
		18	5.5	1.7	12.5	1.7	1.5		Clear area
Fig. 3		24	5.5	1.7	12.5	1.5	1.3	1.32	Prespore
Fig. 4		30	5.5	1.7	12.5	1.6	1.3	1.15	Prespore
		36	5.5	1.7	12.5	1.6	1.3	1.15	Prespore
D	15	45	5.3	1.3	7.0	1.9	1.3		Clear area
	16	3	5.3	1.3	7.0	2.0	1.2		Clear area
		9	5.3	1.3	7.0	2.6	1.2	1.96	Prespore
		15	5.3	1.3	7.0	1.9	1.2	1.43	Prespore
		21	5.3	1.3	7.0	1.5	1.1	0.97	Prespore
	17	33	5.3	1.3	7.0	1.3	1.2	0.98	Prespore
		45	5.3	1.3	7.0	1.3	0.9	0.55	Spore
	19	27	?	?	?	1.3	0.9	0.55	Spore
	21	33	?	?	?	1.3	0.9	0.55	Spore
E	15	15	4.5	1.3	6.0	1.3	1.2	0.98	Prespore
		21	4.5	1.3	6.0	1.7	1.1	1.10	Prespore
		27	4.5	1.3	6.0	1.6	0.9	0.68	Prespore
		51	4.5	1.3	6.0	1.7	0.8	0.57	Prespore
	16	21	4.5	1.3	6.0	1.5	0.8	0.50	Prespore

TABLE 1—*Concluded*

CELL NUMBER	TIME AFTER INOCULATION		CELL			SPORE			STAGE
			Length	Breadth	Volume	Length	Breadth	Volume	
	hours	minutes	μ	μ	cu μ	μ	μ	cu μ	
E—*Concluded*		45	4.5	1.3	6.0	1.5	0.9	0.64	Prespore
		51	4.5	1.3	6.0	1.5	0.9	0.64	Spore
		57	4.5	1.5	7.9	1.5	0.9	0.64	Spore
	19	3	4.5	1.5	7.9	1.5	0.9	0.64	Spore
	21	33	?	?	?	1.5	0.9	0.64	Spore
F	15	15	6.0	1.4	9.2	1.2	1.3		Clear area
		21	6.0	1.4	9.2	1.3	1.3		Clear area
		27	6.0	1.4	9.2	1.5	1.5	1.77	Prespore
	17	9	6.0	1.4	9.2	1.5	1.2	1.13	Prespore
		15	6.0	1.4	9.2	1.5	1.2	1.13	Prespore
	18	9	6.0	1.5	10.6	1.5	1.2	1.13	Prespore
	19	3	6.0	1.5	10.6	1.6	1.2	1.21	Prespore
		9	6.0	1.5	10.6	1.6	1.1	1.04	Prespore
		15	6.0	1.5	10.6	1.7	1.1	1.10	Prespore
		21	6.0	1.5	10.6	1.5	0.9	0.64	Spore
		27	6.0	1.5	10.6	1.5	0.9	0.64	Spore
	21	33	6.0	1.5	10.6	1.5	0.9	0.64	Spore
G	16	0	4.1	1.5	7.3	1.5	0.8	0.50	
H	18	30	4.4	1.5	7.8	1.5	0.9	0.64	
I	18	30	3.9	1.5	6.9	1.5	0.8	0.50	
J	18	30	4.3	1.5	7.6	1.5	1.1	0.97	
K	18	30	6.0	1.5	10.6	1.5	0.9	0.64	
L	18	30	6.0	1.5	10.6	1.5	1.1	0.97	

We have measured the lengths and breadths and calculated the volumes of 75 free spores in a 6 weeks old culture of this strain of *Bacillus megatherium* for comparison with the sizes of the endospores contained in the rods of culture 14 to 21 hours old. Some of the sets of measurements of free spores with a filar micrometer or ocular micrometer showed considerable variations. The most consistent series showed that the average length was 1.6μ, average breadth 0.8μ and average volume of free spores 0.6 cubic micron.

These are approximately the dimensions and volume of the contained endospores. In some of our measurements and calculations the dimensions and volumes of free spores were larger and a general average of all measurements of free spores gave length, 1.7μ, breadth 1.1μ and volume 1.08 cubic micra. Differences in conditions of growth as well as differences in method of measurement may account for these variations.

SUMMARY AND CONCLUSIONS

Endospore-formation in *Bacillus megatherium* was studied by means of motion photomicrography. The organisms were grown on a cone of 2 per cent agar containing 0.05 per cent Difco peptone under a cover glass. Under these conditions spores were formed normally within fourteen to eighteen hours after the inoculation of the medium. Pictures, taken at the rate of 7 per minute, were used by projection and enlargement to investigate the cytological changes occurring during this process and to measure the cells and the spores at various stages.

Some of the factors influencing spore-formation are described and discussed. We are of the opinion that oxygen tension is a more important factor than exhaustion of the medium or accumulation of waste products. Apparently certain optimum conditions are required for spore-formation. Since unfavorable conditions inhibit spore-formation, it seems incorrect to regard the spore as a structure developed for the purpose of resisting an unfavorable environment.

The process of spore-formation in these unstained preparations of *Bacillus megatherium* took place by an apparent condensation of protoplasm in the ends of the cells and a rapid contraction of the material in this region. Cleared areas of dense material in the ends of the cells persisted for two to three hours and then contracted within six to twelve minutes into the highly refractile spore.

Granules in active brownian movement throughout the cells were gradually displaced from these dense clear areas and were unable to penetrate the region in which the spore was forming. There was no visible evidence of the development of the spore from a granule.

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PLATE 1

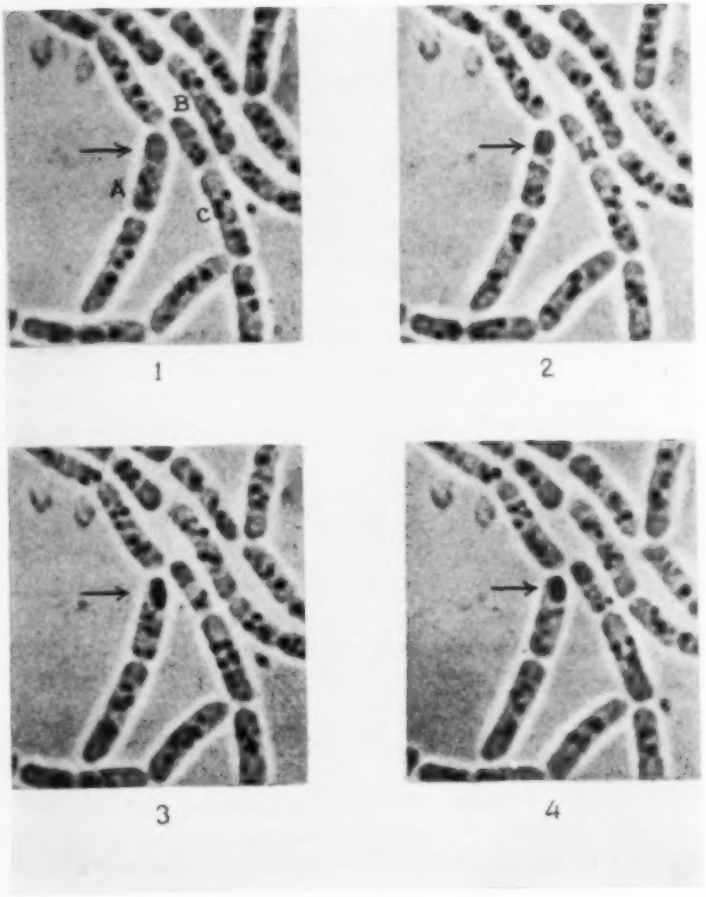
PRINTS FROM SINGLE FRAMES OF FILMS NO. XX, MADE FEBRUARY 5, 1931, SHOWING STAGES IN FORMATION OF ENDOSPORE IN *Bacillus megatherium*. MAGNIFICATION × 3000

FIG. 1. Fourteen hours after inoculation. Prespore in end of cell A. Clear areas in the ends of cells B and C.

FIG. 2. Fourteen hours twelve minutes after inoculation. The prespore in cell A has become denser. The clear area in the end of cell B has become as dense as the prespore in cell A in figure 1. The clear area in the end of cell C has begun to show a definite contour. The pattern of the granules in these cells has changed during the first twelve minutes, but no granules have entered the cleared areas.

FIG. 3. Fourteen hours twenty-four minutes after inoculation. The spore in cell A has a definite elongated ellipsoidal form and is dense and refractile. The prespore in cell B has increased in density. The shape of the cleared area in cell C has changed. Positions of granules in all these cells have changed.

FIG. 4. Fourteen hours thirty minutes after inoculation. In the six minutes between figures 3 and 4, the spore in cell A has contracted to its final form, approaching a sphere, though still ellipsoidal. The prespore in cell B has become denser and the cleared area at the end of cell C has changed its shape somewhat. The pattern of the granules has been altered by movement of the granules in the cells, but no granules have entered the cleared sporogenous areas.



(Stanhope Bayne-Jones and Alexander Petrilli: Formation of the Endospore in B. megatherium)

STUDIES OF FRESHWATER BACTERIA

I. A DIRECT MICROSCOPIC TECHNIQUE

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Received for publication, June 9, 1932

It has long been known that the standard methods of bacteriology—pure culture isolation and observation upon artificial media—often yield only an incomplete knowledge of a particular microbic flora. Both in numbers of individuals and numbers of species, plate cultures from mouth, intestines, or vagina fall far below expectations based upon direct microscopic examination, and the same is often true of saprophytic habitats as well. This fact has been explained in part by the assumption that many of the organisms observed in smears are dead, more satisfactorily on the ground that many of them are incapable of growing upon the artificial media so far devised. From time to time we succeed in cultivating organisms which were previously known only in their natural habitat, but such accomplishments become progressively rarer. It is the author's opinion that only a minor portion of the species of bacteria existing have been cultivated and described.

Soil microbiologists have succeeded perhaps better than any other group in bringing a wide variety of organisms under artificial cultivation. The number of bacterial forms which may be isolated from soil seems to be limited only by the imagination and ingenuity of the investigators in concocting special media having a selective action. But here also one finds that after summarizing all of the information obtained by such pure culture studies, one is left without any adequate conception of the soil microflora as a whole. It is not possible, from the bacteriological analysis, to synthesize a picture of the various organisms living

and working together in their natural habitat. In recognition of this fact one finds in the recent publications of Conn, Winogradsky, and Cholodny, the beginnings, perhaps, of a movement away from the time-honored techniques of bacteriology toward a more rational study of the microbe in its natural environment. A similar development may shortly be expected in other fields of bacteriology.

The movement may in a sense be considered an extension of a movement begun some years ago in the field of general biology which has developed into a new branch of science, ecology. Ecology is essentially a revolt from the analytic and experimental methods of the laboratory, on the ground that these give no adequate conception of the organism as a whole in relation to its environment, and a return to the methods and aims of the old-fashioned field naturalist, freed however from teleologic bias and with the addition, as far as possible, of the precision of the laboratory.

The vast amount of labor that has been spent on water bacteria has been carried on almost exclusively from the standpoint of sanitation and is almost worthless from the standpoint of ecology. We have some information regarding variations in number (as revealed by plate counts) with the seasons, and with the organic content of the water. A certain number of saprophytic species have been isolated on agar or gelatin, described and named, mostly long ago. A few characteristic aquatic bacteria of striking morphology or peculiar physiology, such as *Spirillum volutans*, and the iron and sulphur bacteria, are well known. But these isolated bits of information only serve to emphasize our almost complete lack of knowledge of the types of bacteria characteristically living in fresh water, and the nature of their activities there. One is particularly struck by the brevity and meagerness of the accounts of bacteria in works on freshwater biology, as compared with the rich material available on other groups of organisms. Jordan, writing on bacteria in Ward and Whipple's "Freshwater Biology," states: "There is no special and characteristic class of 'water bacteria,' but germs from the air, from the soil, from decomposing animal and plant substances

and from the healthy and diseased tissues of animals and plants may at times find their way into water."

The present status of the problem has been well summarized by Ruttner in discussing bacteriological investigations at the biological station at Lunz:

> The investigation of the bacterial flora populating our waters has not kept pace with that of other groups of organisms. The reason is doubtless to be sought in the great difficulty which is opposed to a scientific understanding of a very large part of the bacterial flora of the water and the mud. It is true that by means of bacteriological culture methods we have succeeded in obtaining a knowledge of quite a variety of bacterial species that are present in our waters. In the majority of cases these are, however, ubiquitous, readily-growing putrefactive forms that are present everywhere. But many of the typical water bacteria, and especially those groups which are of physiological interest, which play so large a rôle in the chemical transformations of a lake, will not grow on the usual nutritive media which are used in the bacteriological plating procedure. Microscopic observation without the aid of cultural methods is of value only with regard to a few of the larger forms because of the slight morphologic differentiation of the group, and so it happens that our knowledge of the composition as well as the density of the bacterial population of the waters is still extraordinarily fragmentary.

To study the bacterial flora of a lake or pond properly by the usual methods would require an outlay of time and labor and expensive equipment for which sufficient financial support cannot be readily obtained, since the project promises to yield no results of economic importance. This paper is a preliminary communication describing a procedure which requires a minimum of equipment and labor, which will reveal both quantitatively and qualitatively the activity of at least a portion of the aquatic microflora, yielding results that may be readily correlated with the considerable data already available bearing on freshwater ecology, and which may well serve both as a control and a guide for more elaborate studies using pure culture isolation methods.

Some time ago an aquarium in my laboratory developed on its walls a growth of algae. Some microscope slides were placed in the aquarium in the expectation that the algae would also

grow on these and that permanent preparations might be made showing the organisms *in situ*. On removing and staining these slides after a week's immersion, I was surprised to find, in addition to the algae, a thin and uniform coating of bacteria of various forms, some of unusual morphology. This experiment was repeated with other aquaria, with the lily pond in the University greenhouse, and finally with the waters of Lake Alexander, Minnesota. In every case the results were the same.

The deposit of bacteria becomes apparent in a few days and increases progressively, eventually becoming so thick that individual cells may be distinguished with difficulty. That the cells are actually growing upon the glass is indicated by their occurrence in microcolonies of steadily increasing size. They are fairly firmly adherent to the glass, not removed by washing under a tap. In the case of some filamentous forms one may readily see on the slide a "holdfast" by which the organism is cemented at the base. In other cases the groups of cells are evidently surrounded by a sheath of gum which also serves to fasten the colony to the glass. While other organisms, various algae and protozoa, also grow upon the slide, there is no debris of amorphous organic or inorganic matter such as obscures the picture to such a distressing extent in slides prepared from soil by the Conn or Winogradsky techniques. The picture is beautifully clear, and any of the bacterial stains may be used.

These observations have led the author to a conception of the bacterial flora of water different from that held previously. A shallow wayside pool may occasionally develop a pellicle or turbidity due to bacteria, similar to that found in a hay infusion in the laboratory, and one may find abundant bacteria by direct microscopic examination of the surface scum immediately after a lake has "bloomed," and the excess population of blue-green algae is undergoing decomposition. But ordinarily the waters of an unpolluted lake or stream are clear, and few or no bacteria may be demonstrated by direct microscopic examination.[1] On

[1] Snow and Fred found from 740 to 29,300 bacteria per cubic centimeter in microscopic counts of centrifuged water from Lake Mendota, plate counts running from one-seventh to one-fourteenth of the microscopic counts. Microscopic counts of bottom mud will run well into the millions per cubic centimeter.

the other hand, a bit of bottom mud, even from considerable depths, stained by the Conn technique, shows an abundance of organisms comparable with the richest soil. The slime from rocks, the mucous sheaths of colonial algae, scrapings from the leaves of submerged plants, all show a microbic flora rich in numbers and in diversity of forms. It is quite evident that for the most part the water bacteria are not free floating organisms, but grow upon submerged surfaces; they are of the *benthos* rather than the *plancton*.

Microscope slides may be placed in a given aquatic environment for a definite period of time, removed, fixed, stained, and examined. It is proposed by this method to follow variations in the numbers and kinds of bacteria that develop in different sorts of freshwater habitats with the expectation that such studies will provide a clue as to the nature of the activities of the water bacteria and the part they play in the economy of lake or stream.

As far as I can determine, such a procedure has not been used before in the study of water bacteria. Naumann has pointed out that beautiful microscopic preparations of iron bacteria may be obtained by immersing slides for a time in iron-bearing waters, but apparently made no further use of the method. Hentschel has followed the development of various sessile types of aquatic organisms upon slates immersed in streams for varying periods, and clipped slides to these slates for observation of the more minute forms, but makes no mention of bacteria. Thomasson has used a technique apparently identical with that of the author, for following the distribution of diatoms in various habitats, but also makes no mention of bacteria. The precedure is identical in principle with that recently described by Cholodny for the study of soil bacteria.

It was necessary to experiment a little before a satisfactory technique for placing the slides in the water was developed. The slides are attached at intervals to a line suspended from a float and fastened to an anchor at the bottom. Anchors must be of sufficient weight to hold fast in heavy wave action. I have found that a weight less than 2 pounds is insufficient. Satisfactory anchors may be made by fastening a screw-eye in the narrow end

of a small aluminum funnel, which is then filled with melted lead. Floats must be easily visible so that they may be readily found again and will not be struck or snagged by passing boats. I am using one gallon glass jugs. The screw caps are cemented on. Within the jug is a printed sign requesting the curious to leave it undisturbed.

It was necessary to give some thought to the material used for the lines. Close proximity to materials of various sorts would undoubtedly influence the type of organisms that would grow upon the slides. Thus, if one suspended the slides from cotton, linen, or silk lines, these would undoubtedly undergo decomposition, products from which would diffuse through the water and influence the growth of organisms on the glass. The use of iron wire would undoubtedly favor the development of iron bacteria, while copper or other metals would probably exhibit a certain degree of inhibiting effect from the antiseptic action of the metal dissolved in the water. The substance finally decided upon was a rubber covered copper wire. The type of wire used is known as "radio hook-up" wire. It is a flexible, stranded wire of small gauge with a thin soft rubber coating, about 2 mm. in diameter over all.

The slides are fastened to the wire in the following manner: One-inch surgeon's adhesive tape is cut into 2½-inch lengths. After cleaning the slides, one end is heated in a Bunsen flame and applied to the adhesive side of the tape, overlapping the tape for ¼-inch. The tape is then doubled on itself and fastened to the other side of the slide for ¼-inch. The slide is thus held between two layers of adhesive tape, and there extends a tab of this tape (double layer) approximately 1 inch square. A hole is punched through this tab and a number is stamped on the part of the tape which is fastened to the slide, with a rubber stamp. The tab is then dipped in melted paraffin. Slides are fastened to the line with short lengths of the rubber covered wire passed through the hole punched in the tab.

It will be seen from the above that the slides do not come in contact with any elements foreign to the environment except the relatively inert substances, paraffin and rubber. These

undoubtedly will also undergo some decomposition, but it is probable that the rate is so slow that there will be no appreciable effect on the flora developing on the glass. The slides have been fastened to the line at one-meter intervals. Thomasson states that in his diatom studies he found an appreciable difference in the flora developing, with variations of depth as little as 5 cm., and insists that slides should be suspended horizontally if they are to represent the true flora at a given depth. In preliminary observations, no such variations have been encountered in the bacterial flora. If such were the case, one should see an appreciable difference between the two ends of a single slide suspended vertically. This has not been observed.

The slides may be stained by any of the usual bacteriological methods. The clearest preparations have been obtained by simple staining with the ammonium-oxalate crystal-violet solution used in Hucker's modification of Gram's stain. Slides which rest in the bottom mud may have a certain amount of adherent debris, and in some cases Conn's rose bengal method or Winogradsky's erythrosin should be used. Sessile algae and protozoa also develop upon these slides, and the results of the investigation will undoubtedly prove more valuable if some account is taken of these other organisms in relation to the bacteria. They cannot, often, be readily identified from air dried, flame fixed, slides. Slides are therefore placed on the line in duplicates, One is stained with crystal violet for bacteria, the other is fixed in Flemming's solution and stained with Heidenhain's iron haemotoxyline for observation of the algae and protozoa.

The organisms develop upon the slide in a fairly uniform film. If this is of the proper density, the bacteria may be counted with some degree of accuracy. The density increases with the duration of immersion, and is evidently determined by two factors: the number of organisms brought to the slide by water currents or their own motility, and the rate at which they multiply upon the slide. Quantitative studies of the rate of increase of the organisms upon the slide should prove interesting, and will have to be made eventually before more general quantitative studies can be properly evaluated. But preliminary studies at Lake

Alexander indicate that in general slides immersed for two weeks will develop enough organisms to allow a reasonably accurate count, without becoming too dense. This time period will probably have to be varied to suit the season and habitat. Preliminary observations indicate that significant quantitative variations will be noted with the season, the depth of water, and the character of the bottom. The number of bacteria developing upon a unit area of the slide in a unit period of time may serve as a measure of microbic activity in a given environment.

While it is true that bacteria vary too little in morphology to allow of their identification by the microscope alone, I do not feel as pessimistic as Ruttner about the limitations imposed by a purely morphologic study of the water bacteria. A glance at one of the slides prepared by the method described above will astonish one who is not acquainted with these organisms, by the great variety of morphologic types which are present. A few of the larger and well known forms, such as *Spirillum volutans* and *Cladothrix dichotoma*, may be recognized at a glance. But even with the more minute forms, there is a great variation in size, form, and arrangement of the cells, which, if observed systematically, must lead to some orderly understanding of the number and distribution of the species.

Some difficulty has been experienced in deciding whether certain forms encountered are large bacteria or smaller species of *Cyanophyceae*. All of the definite blue-green algae encountered so far have proved to be strongly Gram-positive while nearly all of the definite bacterial forms have been found to be Gram-negative. A few minute spherical bacteria in clusters from bottom mud are Gram-positive, and certain of the larger rod-shaped bacteria show Gram-positive granules within the cells, but in general it has been rather surprising to note the almost complete absence of Gram-positive bacteria. No spore formers have been encountered.

It is obvious that, until more completely known, these water bacteria cannot be fitted into existing classifications, but some sort of a working classification must be adopted in order to analyze the slides intelligently. The forms encountered so far may

be immediately divided into two great groups—those which occur in filaments or chains, and those which occur singly or in irregular aggregates, corresponding to the *Trichobacterinae* and *Haplobacterinae* respectively, of Alfred Fischer. The filamentous types may be further subdivided into those which are continuous and those which are articulated, the latter according to size, shape, and internal structure of the elements. Further noteworthy characters of filamentous types are the presence or absence of sheaths, of true branching, of false branching, and of holdfasts. No true branching filamentous forms have been encountered so far. The non-filamentous types are of widely varying form and size. There are minute spherical forms, singly or in clusters, larger round or oval cells with a reticulated protoplasm much like Azobacter; there are rods of varying length and thickness, some with deep-staining granules, others uniform in their staining. There are comma-shaped cells and true spirilla varying in size. While it is true that one would hardly be justified in naming these organisms and describing them as species on the basis of their morphologic appearance alone, it is quite evident that the morphology is sufficiently diversified to warrant a tentative classification of the organisms observed which will be sufficient for at least preliminary ecologic surveys.

This procedure is not offered as a substitute for pure culture studies. Undoubtedly each of the bacterial forms will, eventually, have to be isolated in pure culture before they may be understood with any degree of completeness. But the method does offer a short cut towards an ecologic survey of the water bacteria. It will indicate what sorts of organisms are to be sought in cultures, and when morphologic types have been correlated with habitats, it may indicate what sorts of media or conditions of cultivation are to be used for their isolation.

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PLATE 1

This collection of photomicrographs is presented to illustrate the wide variety of morphologic types of bacteria which have been encountered. The magnification is 800 times.

Figures 1 to 21 inclusive are filamentous types which the author would classify as trichobacteria; the remainder are haplobacteria. Figures 1 and 2 show the holdfast and the "false" branching, respectively, of *Cladothrix dichotoma*. Articulation or its absence, sheaths or their absence, are illustrated in the other filamentous forms.

Figures 22 to 25 show spiral types. The large spirillum shown in 24 and 25 is probably *S. volutans*.

Figures 33, 34 and 35 show peculiar vibrio types with long attached filaments. These are probably not flagella, as no mordant was used. It is the author's opinion that they are *stalks* by which the bacteria are attached to the glass, similar to the stalks of some diatoms.

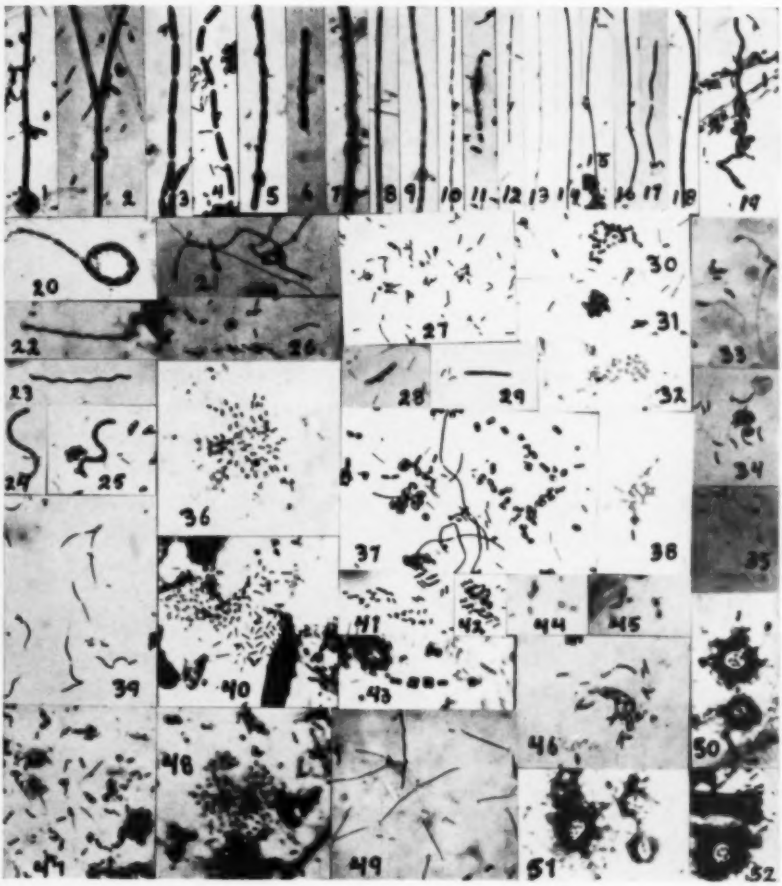
Figures 28 and 29 show rod forms with peculiar faintly stained areas at the poles, probably a plasmolytic phenomenon.

Figure 37 shows large vacuolated oval cells somewhat resembling *Azotobacter*.

Figures 44 and 45 are encapsulated forms.

Figure 47 shows, among others, spindle-shaped types which in form and staining reactions resemble fusiform bacilli of the human mouth.

Figures 50, 51, and 52 show minute colonies of *Siderocapsa major* Molisch. The bacteria are contained within a mucoid capsule, about which ferric hydroxide is deposited in a ring.



(Arthur T. Henrici: Studies of Freshwater Bacteria)

PNEUMOCOCCUS VARIANTS INTERMEDIATE BETWEEN THE S AND R FORMS

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Received for publication, June 8, 1932

Since the publication by Griffith (1923) of experiments dealing with the derivation of an avirulent R form of pneumococcus from a virulent type-specific S form by cultivation of the S form in homologous immune serum,—a phenomenon which had probably been observed but not so extensively studied in the earlier observations of Roger (1891), Neufeld (1902), Friel (1915), and Stryker (1916),—a large number of papers concerned with the S and R forms of pneumococcus have appeared. These have dealt for the most part with the differences in colony appearance, antigenic constitution and virulence of the two forms (Reimann (1925), Amoss (1925), Reimann (1926), Paul (1927)); with the reversion of the R form to the homologous S form (Levinthal (1926), Dawson and Avery (1927), Dawson (1928, 1930)); and with the transformation of R forms into heterologous S forms (Griffith (1928), Neufeld and Levinthal (1928), Reimann (1929), Dawson (1930), Dawson and Sia (1931), Sia and Dawson (1931), Alloway (1932)). Relatively little attention, however, has been paid to intermediate forms which often appear in cultures during the S → R change, forms which may perhaps be of more epidemiological significance than the comparatively stable, avirulent R forms. Brief references to them may be summarized as follows:

Blake and Trask (1923) briefly reported the derivation of three distinct variants from a parent I-S culture by cultivation in homologous immune serum. These variants exhibited changes in colony appearance, agglutinability and virulence by means of which they could be distinguished one from another. None was

a typical R form in colony appearance. Two of these variants, which were stable on prolonged subcultivation, were further reported on by Falk, Jacobson and Gussin (1925) and by Falk and Jacobson (1925), who described differences in the electrophoretic potentials of the variants when compared with that of the original S form.

Yoshioka (1923), by growing virulent Type I pneumococci at 39°C. and by various other modifications of the usual methods of cultivation, derived three variants designated A, B, and C, respectively. The alterations exhibited were diminution of agglutinability in homologous antiserum and the appearance of a new agglutinability in heterologous antiserums. These changes went hand in hand with diminution in virulence but without complete parallelism. Change in agglutinability was already demonstrable in strains which still killed mice in doses of 0.0001 cc. It would seem probable, though not certain, that the variant C, which was fully avirulent, was only partially bile soluble and showed the greatest cross-agglutinability in heterologous antiserums, was an R form and that the A and B variants, which still possessed some virulence and were less readily agglutinable in heterologous antiserums, were intermediate forms.

Paul (1927) described the appearance of daughter colonies in both S and R forms, their development being somewhat earlier and more frequent in ageing S colonies than in R colonies. Although the S daughter colonies appeared not unlike R colonies, it was evident that complete dissociation into an R variant had not taken place, for subculture of these daughter colonies yielded typical S colonies. When S pneumococci were grown on unfavorable media, atypical, small, rough-appearing colonies developed. These were, however, not true R forms for they showed colony autolysis and, if transferred to suitable media, produced typical smooth colonies. Paul designated these as "pseudo-rough" colonies and remarked that the organisms in them might conceivably be closely related to some of the intermediate forms.

Reimann (1929) also noted the occurrence of daughter papillae in recently dissociated R colonies. On subcultivation the daughter papillae yielded shiny, convex colonies. The organisms of

these colonies possessed no capsules, were smaller than either S or R forms, resembled the R forms serologically, and possessed little virulence for mice. He also mentioned briefly a colony form appearing in varying proportions to the R forms after the fifteenth transfer of an original I-S pneumococcus in homologous antiserum broth. This variant was almost indistinguishable from the S form in colony appearance but was avirulent for mice and serologically like the R form.

Dawson (1928), in a paper dealing with the reversion of R to S forms, incidentally mentions the appearance between the fifth and fourteenth transfers of a Type I-S pneumococcus in homologous antiserum broth of numerous peculiar colonies which were neither typical S nor typical R forms. Attempts to isolate and stabilize these intermediate forms were not successful. Similar intermediate forms were not observed during the S → R conversion of Type II and Type III pneumococci.

EXPERIMENTAL

The experiments described below have been carried on at intervals since 1922 in an effort to acquire information concerning pneumococcus variants intermediate between the well characterized S and R forms. In the preliminary experiments briefly reported by Blake and Trask (1923) two stable intermediate variants and a third unstable variant were obtained. Subsequently a second unstable variant but little removed from the S form in its characteristics and two colony types of the unstable form, one of which closely resembled the R form, were recognized. These may have been present but overlooked in the earlier experiments. The terminology adopted for the various intermediates between the S and R forms is as follows: *a*, *b*, *c*, *d*, and *e*. In presenting the experiments it has seemed most convenient to separate them into five headings, even though the observations were carried on more or less concurrently. The first deals with the derivation of the intermediate forms from parent S cultures, the second with their identification by means of colony morphology and agglutination reactions, the third with the attempts at stabilization of the intermediate forms, the fourth with immunological reactions and the fifth with virulence.

Derivation of intermediate variants from S pneumococci by growth in homologous immune serum broth

Methods. Typical S strains of virulent Type I, Type II and Type III pneumooccci were used. These were subcultured daily in homologous antipneumococcus horse serum meat infusion broth, pH 7.8. In the earlier experiments parallel control lines were subcultured daily in heterologous antipneumococcus serum broth, plain meat-infusion broth, or on rabbit-blood agar plates. Incubation was at 37°C. for eighteen to twenty-four hours. In order to detect the presence of variants in the serial cultures a small platinum loopful of each culture was streaked on the surface of a fresh rabbit-blood agar plate. After incubation at 37°C. for eighteen to twenty-four hours, the plates were studied with a hand lens for the presence of colonies differing in appearance from the original S colonies. A rough estimate of the relative numbers of variant colonies was recorded and well-isolated individual colonies of variants were picked for further identification by means of agglutination tests.

Experiment 1. A stock strain of pneumococcus Type I-S, M.L.D. for white mice 10^{-8} cc., was inoculated into a mouse which was killed when moribund and a single colony from the heart's blood culture was seeded into 5 per cent Type I antipneumococcus serum broth. After twenty-four hours incubation at 37°C. a platinum loopful was streaked on a rabbit-blood agar plate and 0.1 cc. transferred by pipette to a fresh tube of serum broth. This procedure was repeated daily for 25 transplants. After twenty-four hours incubation the blood agar plates were studied for the presence of variant colonies. When variants were present, well isolated individual colonies were picked for subculture on blood agar to be further studied. Parallel controls were similarly carried for 25 transplants in 5 per cent Type II antipneumococcus serum broth and in plain broth. The results are shown in table 1.

Two variant colonies, subsequently classified as intermediate *c*, were recognized in the first culture. These rapidly increased as the S forms diminished, having almost completely replaced the S forms by the sixth culture. In the ninth serial culture S forms had entirely disappeared and another colony form, subsequently designated inter-

mediate *b*, was recognized to be present in about equal numbers with *c*. This form had probably been present earlier but was overlooked. Both *b* and *c* persisted in variable proportions throughout the remaining 16 cultures. No other variants were recognized in this experiment. No variants occurred in the control series.

Experiment 2. The same procedure with the same strain of pneumococcus Type I-S was carried out as in experiment 1. In addition

TABLE 1

Derivation of pneumococcus Type I intermediates b and c from Type I-S by growth in 5 per cent Type I immune serum broth

SERIAL CULTURE NUMBER	APPROXIMATE RATIO OF COLONY FORMS ON BLOOD AGAR PLATE CULTURES						
	S	Intermediate forms					R
		a	*b*	*c*	*d*	*e*	
1	+	–	–	2 colonies	–	–	–
2	+	–	–	3 colonies	–	–	–
3	2	–	–	1	–	–	–
4–5	1	–	–	4	–	–	–
6	1	–	–	20	–	–	–
7	1	–	–	6	–	–	–
8	1	–	–	50	–	–	–
9	–	–	2	1	–	–	–
10	–	–	1	1	–	–	–
11	–	–	1	2	–	–	–
12–15	–	–	1	5	–	–	–
16	–	–	1	1	–	–	–
17	–	–	5	1	–	–	–
18–24	–	–	1	2	–	–	–
25	–	–	1	4	–	–	–

+ = innumerable. Control series in 5 per cent Type II serum broth and plain broth remained pure I-S.

Type I-*b* and Type I-*c* strains isolated from the twenty-first serial culture of experiment 1 were grown in serial cultures of 5 per cent Type I serum broth. The results are shown in table 2.

In the Type I-S line S type colonies rapidly diminished and had disappeared by the sixth culture. *b* type colonies appeared in the second culture, reached their maximum in the sixth and had disappeared by the twelfth. *c* type colonies were first recognized in the sixth culture. They rapidly increased in number and remained present until the end of the experiment. A new colony type, designated

intermediate *d* appeared in the twelfth culture and increased in proportion to *c* colonies to the end of the experiment. In the Type I-*b* series *c* colonies appeared in the ninth culture, *d* colonies in the eighteenth. In the Type I-*c* series *d* colonies appeared in the tenth culture. In both of these series the change was in the direction of R with no reversion toward S. The control series remained unchanged throughout.

TABLE 2

Dissociation of pneumococcus Type I-S, Type I-b and Type I-c by growth in 5 per cent Type I immune serum broth

SERIAL CULTURE NUMBER	APPROXIMATE RATIO OF COLONY FORMS ON BLOOD AGAR PLATE CULTURES																					
	Type I-S							Type I-*b*							Type I-*c*							
	S	*a*	*b*	*c*	*d*	*e*	R	S	*a*	*b*	*c*	*d*	*e*	R	S	*a*	*b*	*c*	*d*	*e*	R
1	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
2	5	–	1	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
3–5	1	–	1	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
6	–	–	50	1	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
7	–	–	1	2	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
8	–	–	5	1	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
9	–	–	1	3	–	–	–	–	–	1	1	–	–	–	–	–	–	+	?	–	–
10–11	–	–	1	1	–	–	–	–	–	1	3	–	–	–	–	–	–	50	1	–	–
12	–	–	–	30	1	–	–	–	–	1	30	–	–	–	–	–	–	30	1	–	–
13	–	–	–	10	1	–	–	–	–	1	30	–	–	–	–	–	–	6	1	–	–
14	–	–	–	10	1	–	–	–	–	1	10	–	–	–	–	–	–	8	1	–	–
15	–	–	–	2	1	–	–	–	–	1	3	–	–	–	–	–	–	2	1	–	–
16	–	–	–	5	1	–	–	–	–	1	3	–	–	–	–	–	–	3	2	–	–
17	–	–	–	1	1	–	–	–	–	1	20	?	–	–	–	–	–	5	1	–	–
18	–	–	–	1	5	–	–	–	–	1	50	50	–	–	–	–	–	4	1	–	–

Control lines of Types I-S, I-*b*, and I-*c* in 5 per cent Type II serum broth and plain broth and on blood agar plates showed no dissociation.

Experiment 3. A virulent strain of pneumococcus Type I-S recently isolated from a patient with lobar pneumonia was used. After two mouse passages parallel serial cultures in 20, 5, and 1.25 per cent Type I antipneumococcus serum broth with control cultures in 5 per cent Type II and 5 per cent Type III serum broth were carried for 8 passages, transplants being made daily. The results are shown in table 3.

A new colony type, intermediate *a*, closely resembling the typical S form was recognized for the first time in this experiment. As in the

preceding experiments *b* and *c* forms appeared in the 5 per cent Type I serum broth while the S forms diminished and disappeared. In the stronger 20 per cent serum broth S forms also disappeared and *a* and *b* forms appeared but no *c* forms were detected, while in the weak 1.25 per cent serum broth and in the controls no variants appeared.

Experiment 4. A strain of pneumococcus Type I-S, which had been passed through 20 mice following isolation from a patient with pneumonia, and Type I antipneumococcus serum broth were used. Transplants were made daily for a period of three weeks, blood agar plate subcultures being made from each serum broth culture as in the

TABLE 3

Dissociation of pneumococcus Type I-S in 20 per cent, 5 per cent, and 1.25 per cent homologous immune serum broth

SERIAL CULTURE NUMBER	APPROXIMATE RATIO OF COLONY FORMS ON BLOOD AGAR PLATE CULTURES																						
	20 per cent Type I serum							5 per cent Type I serum							1.25 per cent Type I serum								
	S	*a*	*b*	*c*	*d*	*e*	R	S	*a*	*b*	*c*	*d*	*e*	R	S	*a*	*b*	*c*	*d*	*e*	R		
1	+	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−		
2	1	10	1	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−		
3	1	10	1	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−		
4	−	1	5	−	−	−	−	1	2	5	−	−	−	−	+	−	−	−	−	−	−		
5	−	1	5	−	−	−	−	1	−	−	1	−	−	−	+	−	−	−	−	−	−		
6	1	1	2	−	−	−	−	1	1	−	−	−	−	−	+	−	−	−	−	−	−		
7	1	2	2	−	−	−	−	−	5	5	1	−	−	−	+	−	−	−	−	−	−		
8	−	1	8	−	−	−	−	−	1	6	3	−	−	−	+	−	−	−	−	−	−		

Control series in 5 per cent Type II serum broth and 5 per cent Type III serum broth remained pure I-S.

preceding experiments. Cultures 1 to 5 were in 10 per cent serum broth, 6 to 21 in 20 per cent. The serum was of lower titer than in the preceding experiments. The results are shown in table 4.

In the first 5 cultures in 10 per cent serum broth no variants appeared. In the sixth culture in 20 per cent serum broth there was an abrupt change, S colonies having been largely replaced by *c* and *d* colonies, the latter predominating. In the eighth culture S colonies had disappeared. A few *c* colonies persisted until the eighteenth culture and *d* colonies until the end of the experiment. In the sixteenth culture a new colony form, *e*, appeared. This also persisted till the end of the experiment.

Experiment 5. A stock strain of pneumococcus Type II-S, M.L.D.

for white mice 10^{-8} cc., was carried through thirty-two daily transfers in Type II antipneumococcus serum broth. Cultures 1 to 9 were 10 per cent serum, 10 to 17, 20 per cent serum, 18 to 26, 33⅓ per cent serum, 27 to 32, 50 per cent serum. No variants were detected until the twenty-sixth culture when there was an abrupt change with *c* type colonies almost completely replacing the S forms. In the twenty-seventh culture no S forms were detected and for the remainder of the experiment only *c* forms were found.

TABLE 4

Dissociation of pneumococcus Type I-S by growth in homologous immune serum broth

SERIAL CULTURE NUMBER	APPROXIMATE PROPORTIONS OF COLONY FORMS ON BLOOD AGAR PLATE CULTURES						
	S	*a*	*b*	*c*	*d*	*e*	R
1–5*	++++	–	–	–	–	–	–
6–7†	±	–	–	+	+++	–	–
8	–	–	–	+	+++	–	–
9–12	–	–	–	±	+++	–	–
13	–	–	–	+	+++	–	–
14	–	–	–	±	++++	–	–
15	–	–	–	–	++++	–	–
16–17	–	–	–	±	+++	+	–
18	–	–	–	–	+++	+	–
19	–	–	–	–	+	+++	–
20	–	–	–	–	+++	+	–
21	–	–	–	–	++	++	–

* 1–5 = 10 per cent serum broth.
† 6–21 = 20 per cent serum broth.

Experiment 6. This experiment was similar to experiment 5 except that pneumococcus Type III-S and Type III antipneumococcus serum broth were used. Twenty-six transplants were made, 1 to 5 in 10 per cent serum broth, 6 to 13 in 20 per cent, 14 to 22 in 33⅓ per cent, and 23 to 26 in 50 per cent. No variants appeared.

Identification of intermediate variants

The recognition of variants was accomplished primarily by differences in appearance of surface colonies on rabbit-blood agar plates after eighteen to twenty-four hours incubation at 37°C. The features studied in distinguishing between different colony

forms were size, contour, surface, color, and the presence or absence of hemolysis. As the experiments described above indicate, increasing experience led to an increasing ability to recognize variant forms, some of which were probably overlooked at the beginning. Eventually it became possible to recognize readily the *b*, *d*, and *e* variants with little chance of error. The *a* colony on the other hand so closely resembled the S colony that it could be recognized only when S colonies were present on the same plate, providing an opportunity for direct comparison. Even then the presumptive *a* colonies had to be checked by agglutination tests, which are described below, to establish their identity. The *c* colonies also resembled the S colonies, though less closely. While they could be readily distinguished from S colonies when the two were growing side by side on the same plate, their differentiation when grown in pure culture without S colonies on the same plate for direct comparison was sometimes difficult and agglutination tests were required to establish their identity.

The outstanding characteristics of the variant colonies at eighteen to twenty-four hours incubation are presented below. It should be pointed out that the features described naturally vary somewhat with different lots of media, with the amount of moisture in the medium, with the density of seeding, with the duration of incubation and perhaps with other unrecognized environmental factors which are inevitable and difficult to control in studies of this kind.

The *a* colony is slightly smaller than the S, it is dome-shaped, the surface is moist and smooth, the color a more grayish green than that of the S and there is no hemolysis.

The *b* colony is smaller than the S, and the *a*, its contour is that of a disc, the surface is smooth and moderately moist, the color a gray-green, and there is no hemolysis.

The *c* colony is still smaller, being ordinarily about one-half the size of an S colony. Its contour is domed or convex, the surface is moist and smooth, the color is a fairly dark green, and there is no hemolysis or only slight hemolysis.

The *d* colony is very small and slightly convex, its surface is

either smooth or slightly granular, the color is light green and there is a fairly wide zone of hemolysis.

The *e* colony is about the same size as the *c;* it is usually convex but may show umbilication at twenty-four hours, its surface is usually smooth but sometimes slightly granular, the color is green to brownish green, and there is a narrow zone of hemolysis. This intermediate closely approaches the R form and perhaps should be classified as such, even though the surface of the colony does not appear rough as does that of the typical R form.

The naked eye appearance of forms S, *b*, *c*, *d*, and *e* are shown in figures 1 and 2.

Since it seemed necessary early in the study to devise some means of readily checking the identity of variant colonies as tentatively determined by their appearance on blood agar plates, agglutination tests in homologous and heterologous antipneumococcus horse serum were tried and proved eminently satisfactory. The technique employed consisted in picking a well isolated colony with a platinum loop, emulsifying it in 0.1 cc. of broth, and then transferring a loopful of the emulsion to each of a series of small test tubes containing increasing dilutions of homologous and heterologous antipneumococcus serum in plain meat-infusion broth. The tubes were incubated at 37°C. over night and then read. In studying the agglutination reactions three features were observed, namely, (1) the titer in homologous serum with record of the prozone, the zone of maximum agglutination and the end point, (2), extent of agglutination in heterologous serum, and (3) character of agglutination. By means of these tests it was found possible to substantiate the recognition of variant colonies since S, *a*, *b*, and *c*, colonies each gave characteristic agglutination reactions which could easily be distinguished one from another. The *d* and *e* variants, however, gave results very similar to those of the *c* variant but this offered no difficulty, since they could easily be identified by colony appearance alone. The *d* and *e* variants agglutinated like R forms. The results in a very large number of tests were consistent with any given lot of serum. There was, of course, a shift one way or the other, in the position of the zone of maximum agglutination with different lots of serum

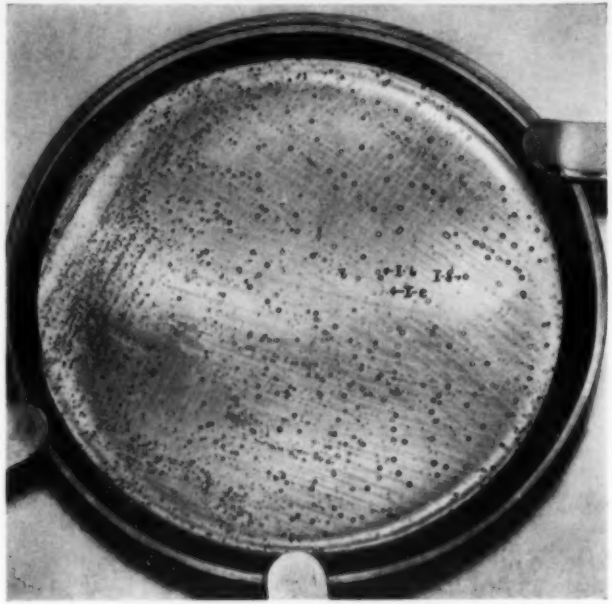

FIG. 1

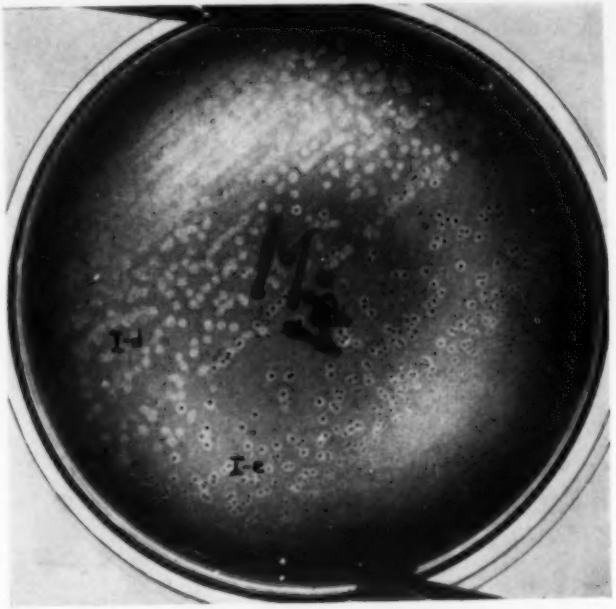

FIG. 2

FIG. 1. BLOOD AGAR PLATE CULTURE SHOWING NAKED EYE APPEARANCE OF PNEUMOCOCCUS TYPE I-S, TYPE I-*b*, AND TYPE I-*c* COLONIES

Twenty-four hours incubation at 37°C.

FIG. 2. BLOOD AGAR PLATE CULTURE SHOWING NAKED EYE APPEARANCE OF PNEUMOCOCCUS TYPE I-*d* AND TYPE I-*c* COLONIES

Twenty-four hours incubation at 37°C.

of different titers, but this was always in the same direction for all the variants. The agglutination reactions are shown in table 5.

TABLE 5

Agglutination reactions of pneumococcus variants in homologous and heterologous immune serums—thread test technique

PNEUMOCOCCUS	SERUM	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	C
Type I-S	Type I	+	±±	++	++	++	+	±	−	−	−	−	−
	Type II	−	−	−	−	−	−	−	−	−	−	−	−
	Type III	−	−	−	−	−	−	−	−	−	−	−	−
Type I-*a*	Type I	+	+	±±	±±	++	++	+	±	−	−	−	−
	Type II	−	−	−	−	−	−	−	−	−	−	−	−
	Type III	−	−	−	−	−	−	−	−	−	−	−	−
Type I-*b*	Type I	±	+	+	+	±±	±±	±±	+	±	−	−	−
	Type II	+	+	±	−	−	−	−	−	−	−	−	−
	Type III	+	±	−	−	−	−	−	−	−	−	−	−
Type I-*c*	Type I	±	±	±	±	±	±	±	+	+	+	±	−
	Type II	±	±	±	±	±	±	±	±	±	−	−	−
	Type III	±	±	±	±	±	±	±	±	−	−	−	−
Types I-*d*, *e*, R	Type I	±	±	±	±	±	±	±	±	±	±	±	−
	Type II	±	±	±	±	±	±	±	±	±	−	−	−
	Type III	±	±	±	±	±	±	±	±	−	−	−	−

++ = compact disc formation; ±± = coarse, fairly compact agglutination; + = medium floccular agglutination; ± fine agglutination; − no agglutination. Type I serum was a high titer therapeutic serum, Types II and III were lower titer diagnostic serums. Complete sedimentation with clear supernatant in all tubes showing agglutination. Diffuse growth in tubes showing no agglutination. C = plain broth control.

Stabilization of intermediate variants

Efforts were made to stabilize each of the variants by repeated daily transplants on blood agar plates, picking a single well isolated colony each time for transfer. If the line appeared to be breeding true after a reasonable number of transplants on blood agar it was then transferred to rabbit-blood broth and subcultured at intervals varying from one to six weeks, the cultures

being placed in the ice-box after eighteen-hours incubation. Stabilization was easily accomplished in the case of Type I-*b*, Type I-*c*, and Type II-*c* forms, which bred true from the start and can apparently be carried indefinitely in blood media without change in colony form, agglutination characteristics and virulence. One example will suffice as an illustration. Type I-*b* and Type I-*c* colonies were picked from the blood agar plate which had been seeded from the twenty-first Type I serum broth culture of

TABLE 6

Agglutination and virulence tests with pneumococcus Type I-b and Type I-c when isolated and after two years in culture

PNEUMOCOCCUS	DATE	SERUM	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
Type I-*b*	3/6/23	Type I	+	+	±±	±±	±±	+	±	−	−
	1/9/25	Type I	+	+	±±	±±	±±	±	−	−	−
Type I-*c*	3/6/23	Type I	±	±	±	±	±	+	+	+	±
	1/9/25	Type I	±	±	±	±	±	±	+	+	+

PNEUMOCOCCUS	DATE	DOSE	0.5 cc.	0.1 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.00001 cc.	HEART'S BLOOD CULTURE FROM MICE THAT DIED
Type I-*b*	3/ 7/23	Virulence for white mice	D 16 hrs.	D 17 hrs.	D 19 hrs.	S	S	S	I-*b*
	1/10/25		D 21 hrs.	D 40 hrs.	D 46 hrs.	S	S	S	I-*b*
Type I-*c*	3/ 7/23		D 7 days	S	S	S	S	S	I-S
	1/10/25		D 62 hrs.	D 69 hrs.	S	S	S	S	I-S

experiment 1 and transferred daily for three weeks on blood agar plates. Since both lines were apparently breeding true they were then transferred to blood broth in which they were kept for approximately two years. Agglutination and virulence tests at the beginning and after nearly two years in culture are shown in table 6.

Attempts to stabilize *a* forms were unsuccessful as they more or less promptly reverted to S forms when cultured on blood agar plates. The *d* and *e* variants likewise could not be permanently

stabilized in pure culture for any considerable length of time. The *d* variant showed a tendency to dissociate in both directions, *c* and *e* colonies suddenly and unexpectedly appearing on the plates at irregular intervals. The *e* variant showed a similar phenomenon, *c*, *d* and R colonies appearing at irregular intervals. Efforts to obtain permanent pure line strains of these variants were abandoned after subculturing for a period of seven months had failed to establish them.

Immunological reactions of variants

The agglutination of *a*, *b*, *c*, *d*, and *e* variants in homologous and heterologous antipneumococcus horse serum has already been shown in table 5. It will be seen that there is an orderly and progressive change in the agglutination reactions proceeding from S to *c* in all the aspects studied and that *c*, *d*, and *e* variants agglutinate like R strains except for the tendency shown by *c* strains to exhibit a zone of maximum agglutination at high dilutions. The obvious suggestion arises that this change, in so far as agglutination in homologous serum is concerned, is probably due to a progressive decrease in type specificity associated with a progressive diminution in capacity to elaborate the specific soluble carbohydrate. Beginning with variant *b* the character of the agglutination changes and agglutination in heterologous serum appears. Again, the suggestion arises that these changes are associated with an increasing opportunity for the common species antigen-antibody reaction to occur as the type specificity of the variants progressively diminishes and disappears. While the foregoing suggestions seem probable, it is obvious that a quantitative chemical analysis of the antigenic constituents of each of the variants would be necessary to establish the fact and this has not been carried out.

The results of agglutinin absorption experiments with Type I-S, I-*b*, and I-*c* forms and Type I antipneumococcus serum, however, lend support to the view expressed. The result of one experiment is shown in table 7. Type I serum completely absorbed of the type specific agglutinins by Type I-S still retains some fine flaking agglutinins for I-*b* and I-*c* though the titer has been reduced

for both. Serum absorbed by I-*b* possibly shows a small amount of type-specific agglutinins remaining and some reduction in fine flaking agglutinins. Serum absorbed once with I-*c* shows considerable reduction in type-specific agglutinins, practically none for the species agglutinins. Following a second absorption the type-specific agglutinins have nearly if not entirely disappeared, and there has been a considerable reduction in fine flaking agglu-

TABLE 7

Agglutinin absorption of Type I serum by pneumococcus Type I-S, Type I-b, and Type I-c

TYPE I SERUM ABSORBED BY	PNEUMOCOCCUS VARIANTS	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
Type I-S	Type I-S	−	−	−	−	−	−	−	−	−
	Type I-*b*	±	±	−	−	−	−	−	−	−
	Type I-*c*	±	±	±	±	±	±	±	−	−
Type I-*b*	Type I-S	±	−	−	−	−	−	−	−	−
	Type I-*b*	±	±	±	−	−	−	−	−	−
	Type I-*c*	±	±	±	±	±	±	±	−	−
Type I-*c* (first absorption)	Type I-S	+	±	−	−	−	−	−	−	−
	Type I-*b*	±	±±	±±	±	±	−	−	−	−
	Type I-*c*	±	±	±	±	±	±	±	±	−
Type I-*c* (second absorption)	Type I-S	±	−	−	−	−	−	−	−	−
	Type I-*b*	±	±	−	−	−	−	−	−	−
	Type I-*c*	±	±	±	±	±	−	−	−	−
Unabsorbed	Type I-S	++	++	+	±	−	−	−	−	−
	Type I-*b*	+	+	±±	±±	+	±	−	−	−
	Type I-*c*	±	±	±	±	±	+	+	±	±

++ = compact disc formation; ±± = coarse, fairly compact agglutination; + = medium floccular agglutination; ± fine agglutination; − no agglutination.

tinins. It would appear from the above that absorption with the S strain removes the type-specific agglutinins leaving the species agglutinins but little effected, that a single absorption with the *b* strain also removes type-specific agglutinins, though less effectively than the S, and also leaves the fine flaking agglutinins but little effected, and finally that absorption by the *c* strain first removes the type specific agglutinins before the species agglutinins are removed.

Another agglutinin absorption experiment, in which a normal human serum containing species agglutinins but no type-specific agglutinins was absorbed by Type I-S and Type I-*c*, supports this view (table 8). Absorption with I-S failed to remove the fine

TABLE 8

Absorption of adult normal human serum by pneumococcus Type I-S and Type I-c

NORMAL HUMAN SERUM ABSORBED BY	PNEUMOCOCCUS VARIANTS	SERUM DILUTIONS							
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Type I-S	Type I-S	−	−	−	−	−	−	−	−
	Type I-*c*	±	±	±	±	±	±	±	−
Type I-*c*	Type I-S	−	−	−	−	−	−	−	−
	Type I-*c*	−	−	−	−	−	−	−	−
Unabsorbed	Type I-S	−	−	−	−	−	−	−	−
	Type I-*c*	±	±	±	±	±	±	±	−

± = fine agglutination; − = no agglutination.

TABLE 9

Immunity of mice to Type I-S following recovery from intraperitoneal injection of Type I-b, Type I-c and Type I-d Pneumococci

DOSE	INITIAL INJECTIONS 3/7/23			IMMUNITY OF SURVIVORS TO TYPE I-S 3/21/23		
	Type I-*b*	Type I-*c*	Type I-*d*	Type I-*b* survivors	Type I-*c* survivors	Type I-*d* survivors
cc.						
0.5	D 16 hrs.	D 7 days	D 72 hrs.			
10^{-1}	D 17 hrs.	S	S		S	S
10^{-2}	D 19 hrs.	S	S		S	D 49 hrs.
10^{-3}	S	S	S	S	S	D 60 hrs.
10^{-4}	S	S	S	S	D 142 hrs.	D 60 hrs.
10^{-5}	S	S	S	S	D 44 hrs.	D 80 hrs.

Type I-*b* and I-*d* survivors received 10^{-7} cc. Type I-S, Type I-*c* survivors 10^{-6} cc. Type I-S. Type I-S controls 10^{-6} cc. and 10^{-7} cc. (2 each) died between 36 and 46 hrs.

flaking species agglutinins for I-*c*, while one absorption with I-*c* completely removed them.

The immunizing value of Type I-*b*, I-*c*, and I-*d* forms is illustrated by an experiment shown in table 9. Mice which had survived intraperitoneal inoculation with living cultures of these

TABLE 10

Virulence of pneumococcus variants for white mice

DOSE	TYPE I-S			TYPE I-b			TYPE I-c			TYPE I-d			TYPE I-R	
	Number of mice	Result	H.B.	Number of mice	Result	H.B.	Number of mice	Result	H.B.	Number of mice	Result	H.B.	Number of mice	Result
cc.														
2.0							1	D 1 day	I-c					
1.0							8	D 3 days*	I-S				5	S
0.5				1	D 1 day	I-b	6	3 D 4½ days 3 S	I-S	2	1 D 3 days 1 S	I-S		
10^{-1}	Many	D 48 hrs.	I-S	5	D 1¼ days*	I-b	12	4 D 3 days 8 S	I-S	3	S			
10^{-2}	Many	D 48 hrs.	I-S	5	3 D 1½ days 2 S	I-b	13	2 D 3½ days 11 S	I-S	3	S			
10^{-3}	Many	D 48 hrs.	I-S	5	1 D 8 days 4 S	I-b	11	S		3	S			
10^{-4}	Many	D 48 hrs.	I-S	5	1 D 3 days 4 S	I-S	9	S		3	S			
10^{-5}	Many	D 48 hrs.	I-S	3	S		4	S		2	S			
10^{-6}	Many	D 48 hrs.	I-S	2	S		3	S		1	S			
10^{-7}	Many	D 48 hrs.	I-S											
10^{-8}	Many	D 48 hrs.	I-S											

D = died; S = survived; H.B. = heart's blood culture at autopsy.

* Average time elapsing before death.

variants were reinjected intraperitoneally two weeks after the initial injection with multiple fatal doses of Type I-S. It will be seen from table 9 that Type I-*b* was the most effective in inducing active immunity, Type I-*c* possibly less so, and Type I-*d* even less effective. Similar experiments with Type I-*b*, Type I-*c* and Type II-*c* have given similar results over a number of years.

Virulence of variants

The progressive change in agglutination reactions is fairly closely paralleled by a progressive loss in virulence for white mice. The results of virulence tests performed at intervals over a period of two years are summarized in table 10 with the virulence of S and R strains included for comparison. In addition to the difference in virulence between I-*b* and I-*c* another difference should be noted. When mice succumbed to I-*b* the organisms recovered from the peritoneal exudate and heart's blood were always I-*b*, with the exception of the one mouse which died from a dose of 10^{-4} cc. On the other hand when mice succumbed to I-*c*, the organisms recovered were always I-S, again with the one exception of the mouse which received the large dose of 2 cc. Furthermore, a longer interval between inoculation and death usually occurred with I-*c* than with I-*b*. While the majority of the virulence tests were not done with lines derived from single cells, the same results were obtained when single cell, pure line cultures were used. The virulence of the Type II-*c* form was identical with that of Type I-*c* and it also invariably reverted to the S form in mice that succumbed.

SUMMARY

Five intermediate variants between Type I-S and Type I-R pneumococcus have been detected by growing Type I-S pneumococci in homologous immune serum broth. They progressively appear and disappear in serial cultures. Two of these, I-*b* and I-*c*, were easily stabilized in pure culture. The intermediates between S and R show a progressive and orderly change in their agglutination reactions in homologous and heterologous immune serums. They also show a progressive loss in virulence for white

mice. Only one intermediate, II-*c*, has been derived from Type II-S. It was easily stabilized and is comparable to I-*c* in its agglutination reactions and virulence.

The observations described indicate that the dissociation of S pneumococci by growth in homologous immune serum is not a simple S → R change but that a considerable number of intermediate variants with fairly well defined characteristics appear and disappear during the process.

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THE GRAM STAIN AND DIFFERENTIAL STAINING

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Received for publication, June 22, 1932

The Gram test is the most commonly employed method for differential staining of bacteria. Despite this fact, and the considerable time which has elapsed since its discovery, its mechanism is clouded in doubt. The rôle of iodine in the test and the difference between positives and negatives are still controversial matters. It seems to be generally known that the triphenylmethane dyes form water-insoluble compounds with iodine; but Stearn and Stearn (1930) attribute the fixation of the dye in Gram-positives to an oxidation of the cell material, rather than to the formation of this compound. Burke and Barnes (1929), on the other hand, ascribe the retention of the dye to the increased size of the dye-iodine molecule, and postulate that the essential difference between positives and negatives resides in the lower permeability of the cells of the former type to the compound. This conception is negatived by the observation of Stearn and Stearn that the dye-iodine compound dissociates into its components in alcohol solution. Among other views of the Gram mechanism are those of Brudny (1908) who considers that Gram-negatives are impermeable to iodine; and of Benians (1912), who suggests a lack of permeability of positives to alcohol.

Benians' concept of a difference between positives and negatives in alcohol permeability would explain the so-called "pseudo-Gram" reaction obtained by the use of Victoria Blue and other alcohol-soluble dyes, such as Spirit Blue and Night Blue, if we assume that the dissolved dye molecule unites with several molecules of the solvent. This would involve the passage of a considerable number of solvent molecules into and out of the stained cell. However, since alcohol does extract methyl and

crystal violet from all bacteria, the positivity resulting from iodine treatment still requires explanation. In order to test the solubility of the crystal violet-iodine complex in alcohol, equimolar solutions of the two were mixed and the concentration observed at which a noticeable precipitate was formed. This occurred at a concentration of 0.5 per cent crystal violet.[1] This is about one-tenth the solubility given for gentian violet, a dye mixture comprising methyl and crystal violet. The low solubility of the compound is not incompatible with the observations of Stearn and Stearn previously cited, as to the dissociated state of the dye-iodine compound dissolved in alcohol, and is probably due to an equilibrium between the associated and dissociated states, the former being only slightly soluble. The fact that precipitation leaves a violet supernatant solution, while Gram-stained bacteria are usually blue, can be considered as confirmation of the observations of Stearn and Stearn.

If we consider that the removal of a dye molecule from the interior of a cell requires a driving force supplied by osmotic pressure, and hence proportional to concentration, the reduction of its solubility to one tenth undoubtedly retards extraction. The Gram test, which really amounts to the slowing down of dye extraction by the alcohol, may then be explained as a low permeability of positives to the dye. But with the entrance of alcohol into the cell a solution of iodine is formed as well as one of dye. Consequently, the removal of the dye can only take place if it is accompanied by an escape of iodine; since otherwise the iodine remaining behind would precipitate the dissolved dye in accordance with the mass law. In other words, permeability to both dye and iodine is necessary if decolorization is to take place. While it is true that aqueous Gram iodine solution penetrates Gram-positives as well as negatives (which we have proved by the rapid decolorization of methylene blue stained bacteria by Gram iodine), it must be remembered that the Gram solution contains iodine as the complex ion I_3, whereas it exists in alcohol in molecular form.

[1] A similar test with practically water-free ethyl alcohol showed a solubility below 0.3 per cent. This explains why the Gram test cannot successfully be carried out under anhydrous conditions.

Moreover, the permeability of the cell in water is probably different than in alcohol.

If the above reasoning is valid, retention of the dye by positives may be due not only to a low permeability to the dye, but to a low permeability to iodine as well. As an indication of the importance of the latter factor may be cited our observation that the decolorization of Gram-negatives is prevented by the addition of 0.2 to 0.3 per cent of iodine to the alcohol. Still more striking is the fact that methyl alcohol, which decolorizes most Gram-positives almost instantly, does not, when as little as 0.01 per cent iodine is

TABLE 1

ORGANISM	PER CENT IODINE				
	0.01	0.02	0.04	0.06	0.08
B. subtilis	+	+	+	+	+
M. tetragenus	±	+	+	+	+
Sarcina aurantiaca	−	− − − +	− − − +	+	+
Staph. aureus	−	±	+ + −	+	+
Strep. hemolyticus	−	−	−	−	−
D. pneumoniae	−	−	− − − +	+ + −	+ + −

added, decolorize such positives as *B. subtilis*, *M. tetragenus*, etc., while only slightly larger additions of iodine are necessary to prevent decolorization of other positives.

Table 1 shows the results of tests on six organisms obtained from Bellevue Medical School.[2] A set of slides was prepared, on each of which were placed, side by side, the six organisms. After staining with a 0.5 per cent solution of crystal violet in 2 per cent sodium acetate, they were treated with Gram iodine solution, washed and dried, and then placed in small jars containing various concentrations of iodine in methyl alcohol (C. P. Eimer

[2] These organisms were supplied to us through the kindness of Dr. Wm. H. Park to whom we owe thanks.

and Amend) to which 5 per cent of water was added. Small quantities of freshly prepared and dried calcium carbonate were placed in each jar to insure neutrality of the methanol. The slides were kept in motion in the solutions for two minutes and then promptly washed and counterstained with Safranin for twenty to thirty seconds.

We see from the table that Gram-positive bacteria may be differentiated according to their degree of positivity as measured by iodine concentration in methyl alcohol necessary to prevent extraction of iodine and dye from the cell.

Similar tests with other organisms gave approximately constant values for each species, so long as the water content of the alcohol remained constant. The more aqueous the methanol, the more iodine was required to prevent extraction.

Organisms were frequently encountered which showed varying degrees of positivity in different parts of the cell body. Figure 1 is the photograph of a twelve-hour culture of a soil spore-bearer which had been Gram stained and then treated as above with methyl alcohol containing 0.04 per cent iodine. A strain of *B. diphtheriae* isolated from a clinical case and tested in the same way gave positive terminal metachromatic granules at 0.01 to 0.02 per cent iodine, while the rest of the cell body required much higher iodine concentrations. It is interesting to note that a strain of *B. Hoffmani* isolated from a nasal discharge required 0.05 to 0.06 per cent iodine to prevent dye-iodine escape.

Whether this difference is due to a difference in permeability to dye or to iodine is hard to say off hand, since a decrease in either would probably have the same effect. In order to investigate this point, we exposed crystal violet stained slides, omitting Gram iodine treatment, to ethyl alcohol solutions of iodine, since, under these conditions, iodine and dye move in opposite directions and cell permeabilities to them have opposite effects. For example, table 1 shows *M. tetragenus* to be more highly positive than *Sarcina aurantiaca*. If the greater positivity were ascribable to lower permeability to iodine, one would expect *M. tetragenus* to require the higher concentration of iodine to insure dye retention. The test, however, gave the opposite result. On the other hand,

B. subtilis, which shows the highest degree of positivity, does not retain the dye in all parts of the cell body even when 0.2 per cent iodine has been added to the alcohol, an amount which is sufficient to suppress the escape of dye even from *B. coli*. The arrangement of stained and unstained spots obtained in this way from certain

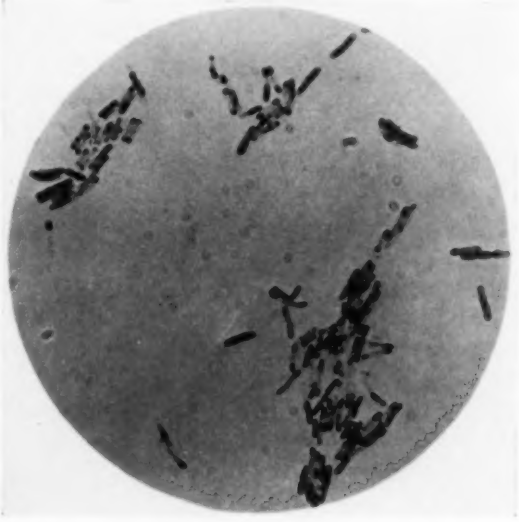

FIG. 1. TWELVE-HOUR CULTURE OF SOIL SPORE BEARER, GRAM-TREATED AND EXPOSED TO THE ACTION OF METHYL ALCOHOL CONTAINING 0.04 PER CENT IODINE

members of the subtilis group is striking. Figures 2 and 3 are photographs of slides of *B. subtilis* and a soil spore bearer, respectively. The slides had been stained with crystal violet and then treated for two minutes with 0.08 per cent cent iodine in 85 per cent alcohol. The unequal behavior of different parts of the cell was also observed in other organisms. Thus, *B. diphtheriae* shows retention of dye in the terminal bodies, while *B. Hoffmani*, in the early transplants after isolation from the human body, showed decolorization on one side and dye retention on the other side of the cell.

FIG. 2. B. SUBTILIS STAINED WITH CRYSTAL VIOLET AND TREATED WITH 0.08 PER CENT IODINE IN 85 PER CENT ETHYL ALCOHOL

Note stained and unstained spots. Compare with figure 3.

FIG. 3. SOIL SPORE BEARER STAINED WITH CRYSTAL VIOLET AND TREATED WITH 0.08 PER CENT IODINE IN 85 PER CENT ETHYL ALCOHOL

These facts point to different degrees of permeability to iodine of the Gram-positives. The comparative resistance of *M. tetragenus* to decolorization by ethyl alcohol containing iodine indicates that its resistance to dye escape is higher than that of Sarcina without a corresponding increased resistance to iodine passage. This shows that the resistance is due not only to the size of the moving molecule but also to factors specific to the cell. Instances of a certain degree of parallelism between the two resistances can be cited, though limited to interior parts of the cell and aqueous iodine solutions. Treatment of bacteria with dilute alkali (0.1 normal NaOH) developed a difference in different parts of the cell which becomes pronounced by either of the following staining methods: (1) Staining with methylene blue and then covering with Gram iodine for one minute; (2) staining with crystal violet and counterstaining with safranin for two minutes.

Figures 4 and 5 illustrate the first method applied to *B. subtilis* and a soil spore-bearer respectively; and figures 6 and 7 show the same organisms treated by the second method. The similarities of the two photographs of each organism are striking, since the dark bodies in the photographs obtained by the first method are due to the failure of iodine to penetrate to those parts, while in those obtained by the second method they are due to the failure of crystal violet to escape from those parts. We see here examples of parallelism between the two resistances. Another example is the strain of *B. diphtheriae* mentioned above which, when stained by these two methods without previous alkali treatment, gave good pictures of the terminal metachromatic granules (see figure 8, from a photograph after staining this organism with crystal violet and counterstaining with safranin for two minutes). These granules also appear when Gram stained slides are treated with iodine in methyl alcohol.

Such parallelisms, however, do not exclude the possibility of divergence. The behavior of Gram-negatives may be regarded as an extreme case. When suspended in water they often exhibit greater resistance to dye escape than many positives. *B. coli* stained with crystal violet and counterstained with safranin (dye displacement method called method 2 above) lost its crystal

FIG. 4. B. SUBTILIS TREATED WITH 0.1 N NaOH, STAINED WITH METHYLENE BLUE, AND DECOLORIZED WITH GRAM IODINE SOLUTION

FIG. 5. SOIL ORGANISM TREATED WITH 0.1 N NaOH, STAINED WITH METHYLENE BLUE, AND DECOLORIZED WITH GRAM IODINE SOLUTION

FIG. 6. B. SUBTILIS TREATED WITH 0.1 N NaOH, STAINED WITH CRYSTAL VIOLET AND COUNTERSTAINED WITH SAFRANIN FOR TWO MINUTES

FIG. 7. SOIL SPORE BEARER TREATED WITH 0.1 N NaOH, STAINED WITH CRYSTAL VIOLET, AND COUNTERSTAINED WITH SAFRANIN FOR TWO MINUTES

violet less readily than Sarcina and many soil spore bearers. Since the quantity of the counterstain is great compared to the originally absorbed dye, preferential affinities are not likely to play any rôle. We can conclude, therefore, that *B. coli* offers considerable resistance to dye passage. Since *B. coli* and other Gram-negatives stained with crystal violet and treated with iodine according to the Gram method show remarkable acid-fastness, the

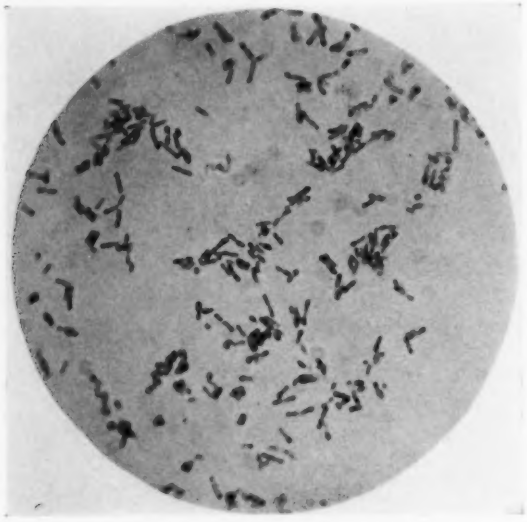

FIG. 8. B. DIPHTHERIAE STAINED WITH CRYSTAL VIOLET AND COUNTERSTAINED WITH SAFRANIN FOR TWO MINUTES

possibility of a failure of iodine to enter, as suggested by Brudny, is excluded. From this we conclude that Gram negativity is due to low resistance to iodine escape (that is, high permeability to iodine). Attempts to measure this resistance by iodine back-pressure, so effective in the case of the Gram-positives, did not give clearly marked results. *B. coli* and others tested with low iodine concentrations in ethyl alcohol showed retention of the dye only in a thin line in the interior of the cell. As the iodine concen-

tration is increased, the line grows in thickness until it reaches the outer visible border of the cell at about 0.2 per cent iodine. Many Gram-positives when subjected to the action of methyl alcohol containing iodine in insufficient amount to prevent the escape of the dye completely, show, in contrast to the Gram-negatives, some decolored cells and some cells in which the dye is retained. The lack of a sharp transition in the case of the negatives, and the contrasting behavior of the positives, gives the impression that the latter have a limiting membrane of higher resistance or, to use Churchman's phraseology (1929), a cortex, which the Gram-negatives lack.

Another indication of a structural difference between Gram-positives and negatives is the ease with which the latter are dissolved by alkali in contrast to the resistance offered by the former (Smith, 1922). We have observed that even 0.1 N sodium hydroxide converts emulsions of Gram-negatives into slimy masses. Some members of the group, such as *B. typhosus*, Proteus and others have formed slime when treated with alkali as dilute as 0.025 N. We have encountered an exception to this rule in the case of an unidentified borderline organism which formed slime with 0.2 N alkali and yet, in view of its behavior when Gram-treated and immersed in ethyl alcohol containing low concentrations of iodine, should be classed with the positives. Despite this exception, the only one encountered, we consider this slime formation to be a fair test for Gram negativity.

The test is performed by emulsifying scrapings from an agar culture on a slide in a drop of water and mixing a small loopful of the emulsion with an equal quantity of 0.2 N NaOH on another slide. *B. proteus*, *B. typhosus*, and para A and B are converted almost instantly into a heavy, sticky mass; while *B. coli* sometimes requires a few seconds for complete conversion. The alkali treatment usually does not interfere with ordinary staining, and brings about the differentiation of the interior parts which becomes visible as beading when treated by methods 1 or 2 described above. In the cases of *B. coli* treated with quantities of alkali small in proportion to the number of organisms present,

methylene blue and erythrosin are effective in bringing out the beading, the body appearing faintly pink while the beads take on a deep blue.

In general, the combination of a basic dye such as methylene blue and an acid dye such as eosin or erythrosin has been found to possess excellent differentiation possibilities, though dividing bacteria along entirely new lines. Thus, *B. subtilis* and other spore bearers lose the initial blue basic dye and become pink, with occasional round blue bodies in the interior of the cells. Gram-negatives, on the other hand, retain the original blue dye, although *B. proteus* and *B. typhosus* give a mixture of blue and pink cells. All the streptococci we could obtain stained pink; while the staphylococci varied, some staining entirely blue while others showed mixtures of pink and blue cells. Previous treatment of smears with sufficient quantities of alkali resulted in Gram-negatives staining pink exclusively, while blue-staining Gram-positives gave mixtures of blue and pink cells. Acid treatment prior to staining also affects different organisms differently. In short, there seem to be considerable possibilities of differentiating bacteria by means of dye pairs.

It should be noted that the capacity of cells to retain methylene blue after treatment with erythrosin is completely lost if smears are treated with methyl alcohol, which fact points to the removal of some important bacterial constituent from the cell, and eliminates the possibility of fixing with alcohol.

Since most Gram-negative bacteria retain the original blue dye, the method described above should be advantageous in searching for these organisms in smears containing pus. We tried it on a pus-containing nasal discharge of a child sick with the grippe and were able to see numerous small blue, stained bacilli, probably of the influenza type, against a pink background of mucus and pus cells.

Alluring as the prospects of research in this direction appear, the lack of a sufficient number of labelled bacteria and a proper collection of dyes has prevented our continuing along these lines. We are at present investigating the effect of solutions of iodine in other solvents.

SUMMARY

Gram-positives act in a manner indicating a low permeability to iodine in alcoholic solution. This, combined with the low alcohol solubility of the dye-iodine compound, is probably the cause of dye retention by positives in the Gram test. A method is described for measuring the degree of permeability to alcoholic iodine. This makes possible the subdivision of Gram-positives into groups according to their degree of permeability. In contrast to the Gram-negatives, positives are shown to have properties indicating a high resistance to iodine passage along the outer limits of the cell, in harmony with the view of Benians (1919–20). This assumption is further supported by differences in behavior on alkali treatment, which readily converts negatives into slime. Although positives do not form slime under these conditions, both types give rise to beading when treated with alkali. Methods are described for bringing out this beading. Alkali treatment resulting in slime formation does not interfere with ordinary staining reactions. The method described, of studying resistance to dye movement by displacing one basic dye with another, while often differentiating bacteria of the same group, does not show a parallelism between resistance to dye movement and Gram positivity. Remarkable results in differentiation of bacteria along new lines were obtained by the dye replacement method when an acid dye was used to replace a basic dye. These results with dye pairs indicate further possibilities.

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STUDY ON COLLODION MEMBRANE FILTERS

PART I

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Received for publication, June 22, 1932

PREPARATION OF GRADUATED MEMBRANES

The ever-increasing interest in, and comprehension of, the importance of the new field in microbiology, which relates to the so-called ultraviruses and filtrable forms of bacteria, on the one hand, and of the rôle of colloids in the processes of life, on the other, urgently call for further developments in the technique necessary for their study. More adequate methods of filtration, and particularly ultra-filtration (in the widest sense), are especially required. The object of this study is to record certain facts and observations made on the technical side of the preparation of collodion membranes developed during my studies on bacteriophage.

I do not intend to attempt any theoretical discussion or explanation of the facts observed either by others or myself. Want of access at the time to the extensive literature on the subject prevents my entering into such a discussion.

Up to the present we possess no material which we can call ideal for filtering purposes. An ideal filter can be defined as one which will act as a simple sieve alone, i.e., will mechanically retain or let pass particles suspended in a liquid, without changing their physical or chemical nature. In such a case the results of filtration would depend solely on the relative dimensions of the pores of the filter and of the particles suspended.

With the filters at present in use, however, it is very seldom indeed that the finest suspensions can be *mechanically* separated. In the great majority of cases such a separation is produced by a

much more complicated process in which incompletely studied phenomena such as adsorption, coagulation, etc., intervene.

Filters made out of inorganic materials—porcelain, silica, gypsum etc.—are comparatively coarse. Their power of adsorption is great and their porosity can neither be exactly determined nor varied outside certain limits.

The use of animal membranes (amnion, alantois, bladder, etc.) or specially prepared paper membranes is restricted. These can only be used for special purposes, e.g., dialysis. Collodion membranes, on the other hand, offer much wider possibilities.

In the literature on the subject a "collodion membrane" has generally been taken to mean a "collodion sac." In this study the term "membrane" is used to describe any membrane forming a film of more or less permeable material. The term "membrane filter" is reserved for a disc of collodion film used in a special funnel for filtration purposes.

A considerable amount of work on the different uses of collodion sacs and membranes has been published and different methods of preparation have been advised. Some workers have recognised the variability of the membranes obtained and the difficulties encountered in producing membranes of a desired permeability. Many studies in different fields of biology have, however, appeared in which membranes have been used without paying the necessary attention to their properties. A very superficial description of the technique employed for the preparation has sometimes preceded, indicating the solvents and strength of collodion solution alone. Very few authors actually have introduced necessary controls and proved that all the membranes which they have used in their study were of comparable properties.

COLLODION SACS

During my researches on the properties of collodion sacs I came to the conclusion that it was almost impossible to prepare two sacs of equal permeability. Malfitano's method of dipping a test tube in a solution of collodion, taking it out and rotating it until the collodion sets, in particular, produced sacs which could not

bear comparison with each other. The technique of Gates[1] proved to be better in that one could obtain from 4 to 6 sacs of almost the same permeability at one time. But a second series of sacs of a permeability approximate to that of the first could only be obtained by chance.

The cause of such divergent results lies in the extreme sensitiveness of the membrane to the different factors governing the formation of its final structure during the period of drying.

In Malfitano's method the resulting permeability of the sac depends upon the following factors:

1. The composition of the solution used, nature of the solvents, percentage of celloidin, brand of celloidin, etc.

2. The number of times the solution was used (e.g., the amount of evaporation of the solvents which has occurred during previous manipulations).

3. The rapidity with which the tube is withdrawn from the solution, and its rotation until the collodion sets, factors which are responsible for differences in the uniformity of the sac wall.

4. Atmospheric conditions (temperature and humidity) prevailing at the time.

5. The duration of the period of the drying of the sac.

In Gates' method, besides the points already mentioned, the following factors are of importance:

1. The diameter of the tube used.

2. The time the tubes are left above the collodion solution before the cork is removed.

3. The position in which the tubes are left to dry, i.e., whether with the mouth of the tube upwards or downwards.

I have made a very complete study of collodion sacs made by these two methods, but chiefly by that of Gates', and as a result of my experiences have found the following technique to be the best for measuring the comparative permeability of different types of the sacs.

To begin with, the sacs are cut to the length of 6 cm. and mounted on tubes exactly 20 cm. long, in such a manner as to

[1] Jour. Exp. Med., 1922, **35,** 635.

leave free 5 cm. of the length of the sac. They are then filled to the top of the tube with distilled water and their intactness controlled by observing the surface of the sac after carefully drying it with filter paper; should a drop of water appear continually at any particular point on the surface, the sac is rejected.

FIG. 1. PERMEOMETER FOR COLLODION SACS

The sacs are then immersed in distilled water to a depth of exactly 6 cm. By so doing and by keeping the sac and tube filled with water a constant pressure equal to the height of the column of the water in the tube was assured in every case. After about half an hour the permeability of the sac becomes constant.

The permeability of the sacs was then measured by a very simple device (fig. 1). A 0.1 cc. pipette about 25 cm. long is mounted in

an India rubber stopper in such a way that its proximal end just passes through the stopper. A mark is then made on the pipette about 5 cm. from the distal end and a second one 10 cm. further down. Before fitting the cork into the tube it is essential that the latter should be completely filled with water so that as the cork is inserted the water rises and completely fills the pipette also. The level of the water in the pipette immediately starts to fall depending on the permeability of the sac. As soon as the water-level reaches the upper mark a stop-watch is put in motion and the time necessary for the water to reach the lower mark is noted. This expresses the permeability of the sac in seconds.

It is important that this test be carried out with the sac immersed in water, otherwise the flow through the membrane will be irregular, especially with sacs of low permeability.

By means of this test I have found it possible by Gates' technique to obtain a series of from 4 to 6 sacs of similar permeability. The preparation of a new series of sacs of the same permeability as the first series was, however, purely a matter of chance, and that chance never came when it was most wanted. Certain experiments which gave valuable results completely lost their significance for want of confirmation, as it was found impossible to prepare sacs identical with those used in the first experiment.

As the result of my experience with these types of collodion sacs, I have been forced to the conclusion that the uses of such sacs are very limited and that they should not be employed for researches in which the permeability of the membranes is an important factor.

MEMBRANE FILTERS

On the other hand, it is much easier to prepare membrane filters of comparable permeability. It is true, one cannot be certain of preparing an exact replica of a large membrane filter (although several small ones, which will then have equal properties, may be cut out of one large sheet), but by observing the same technique, membrane filters of practically equal properties can be obtained, and their preparation involves much less of the personal element. The permeability of a membrane depends on the following three

factors: (*a*) the composition of the collodion solution; (*b*) atmospheric conditions (temperature and humidity); (*c*) the length of time the membrane takes to dry before being immersed in water.

These three factors are easier to control than all the factors involved in the preparation of sacs.

MATERIAL FOR USE

Collodion is a low nitrated cellulose. Its nitrogen content is not constant and varies with different samples. A highly nitrated cellulose such as pyroxylin or smokeless powder, for instance, cannot be used for the purpose, as it is not completely soluble in alcohol-ether. I advise the use of a nitrocellulose of known make, which is guaranteed to be of more or less constant composition, so that the results will not vary from batch to batch. Different brands of nitrated cellulose vary considerably in adsorptive power. By filtering a 1 per cent dilution of serum in physiological salt solution through a membrane prepared from the collodion under examination and determining the quantity of albumen in the first 10 cc. of filtrate by the addition of 20 per cent sulfosalicylic acid, sufficient information on this point can be obtained. If the reaction is frankly positive, the adsorptive power of the celloidin is low enough; if not, another brand must be obtained. Some of the brands are not sufficiently responsive to the action of the chemicals used to obtain membranes of different permeabilities, and should not be used.

STOCK SOLUTION OF COLLODION

In the present study I have used Schaering's celloidin. This celloidin is commonly employed for histological work, and was the only one obtainable. It is pure nitrocellulose of comparatively constant composition sold in air-tight tins in tablet form. Each tablet contains about 40 grams of nitrocellulose saturated with alcohol so that it weighs more than 100 grams. This brand is not an ideal product, as it still contains a considerable amount of oxycellulose which is not soluble in alcohol-ether. I was not able to get another brand and so my experiments have been confined to this one alone. The stock solution, which in my hands

has proved to be the most convenient for the purpose, has the following composition:

Pure nitrocellulose	40 grams
Alcohol, 96 per cent	100 cc.
Ether	300 cc.

The solution can be prepared directly from the "alcohol" tablets mentioned above or else the tablet can be dried and weighed before dissolving. Experience shows that the two methods give different results; a solution prepared from dry celloidin always gives less permeable membranes. In the first case the whole tablet is cut into thin long slices; small pieces should be avoided, as they get stuck together in a mass and are then difficult to dissolve. The slices when cut are weighed and should weigh 121 grams: 40 grams of cellulose plus 81 grams (the weight of 100 cc. of 96 per cent alcohol). If their weight is more than this they must be quickly dried by fanning. If the total weight is less than 121 grams the slices are put into a tared bottle of 500 cc. capacity, and alcohol (96 per cent) added until the proper weight is obtained. Three hundred cubic centimeters of ether are then added and the bottle shaken, preferably in a shaker. A bottle with a good *cork* stopper must be used. Rubber stoppers are soluble in ether, and glass ones either do not fit close or stick to such an extent that they cannot be taken out.

If dry celloidin is used the tablet is grated through an ordinary kitchen grater or passed repeatedly through a meat grinder. With the latter method it is necessary to keep the celloidin moistened with alcohol to prevent its particles sticking together. The celloidin is then left in a dish, covered with filter paper, until a constant weight is established. The weight is then noted and the necessary amount of alcohol added. After the celloidin is thoroughly soaked in alcohol for a few hours or overnight the ether is added and the bottle shaken in a shaker until solution is complete. It is better to keep nitrocellulose in solution. If exposed to the air in the dry condition, it slowly oxidises to oxycellulose, which is insoluble in the alcohol or ether. This applies also to the tablets of celloidin sold in air-tight tins. When

the tin is opened the whole tablet must be dissolved at once, otherwise it will not give a transparent solution.

PREPARATION OF MEMBRANES

Apparatus necessary

1. A small levelling table. This consists of a board of 50 by 50 cm. with four levelling screws at the corners. The board is painted black to make the air bubbles in the membrane visible,

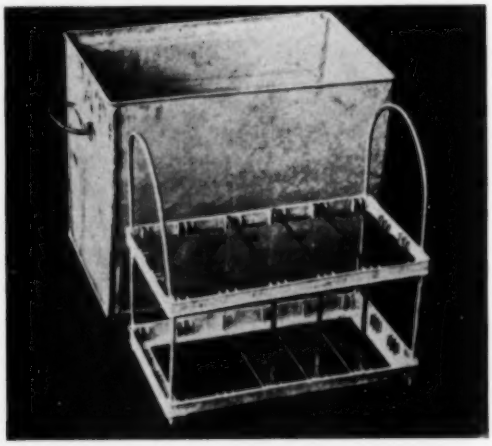

FIG. 2. TANK WITH HOLDER FOR WASHING MEMBRANES

and is covered with a glass plate to insure the flatness of the whole surface.

2. A good spirit level, large and very sensitive.

3. Six flat cast glass plates 20 cm. square and 5 or 6 mm. thick. These must be plane-parallel and must be free from scratches. The best variety is made of the so-called crystal or mirror glass.

4. A copper tank with a holder like those used in photography into which the plates can be put vertically, some 4 or 5 cm. apart, when the membranes on them are ready to put into water (fig. 2).

5. An aluminium stick about 3 mm. thick and 15 cm. long.

6. Thick-walled test tube with 1 cc. graduations up to 15 cc.

7. Cork stoppers of the best quality (velvety), so that no cork dust may pass into the solutions, to fit the test tubes. These must be about 4 cm. long and not too conical.

8. A Petri dish cover of about 90 mm. outside diameter.

9. A small sharp scalpel, or better a safety razor blade in a suitable holder.

10. A few 1 cc. pipettes graduated to 1/100.

SOLUTIONS

1. Ten per cent solution of celloidin.

2. Mixture of 25 parts of 96 per cent alcohol and 125 parts of ether to which about 0.1 per cent of concentrated HCl is added.

3. Acetic ether and formic ether.

TECHNIQUE OF PREPARATIONS OF MEMBRANE FILTERS

The principle of the technique consists in pouring a properly diluted stock celloidin on the glass plate and leaving it for a definite time until the ether evaporates and the celloidin sets. The plate is then put into water to wash out the solvents. A disc can then be cut out of the film, which may be used as a membrane filter in Zsigmondy's funnel.

To change the permeability of the membrane, measured amounts of acetic or formic ether are added to the diluted celloidin solution.

PREPARATION OF THE GLASS PLATES

Before use, the glass plates should be carefully cleaned with acetone and polished with a cloth. However, it was observed that in spite of every care in cleaning the glass, the membranes would sometimes stick to the glass, or sometimes become detached from it too early and shrink without the necessary support. After numerous trials it was found that this was not due to the condition of the glass surface alone, but mainly to the reaction of the celloidin solution; a slightly alkaline film sticks to the glass, while a slightly acid one detaches itself very early. The addition of a very minute quantity of the hydrochloric acid to the alcohol-ether diluting mixture always produced an easily detachable membrane. The maximal amount necessary for this purpose is 0.1 per cent

HCl, and very often considerably less. On the other hand, the premature detachment of the film before the solvents are all washed out introduces another difficulty in the form of ultimate shrinkage and a change in the quality of the membrane, usually in the production of a membrane which is difficult to use owing to its uneven surface. To overcome this latter difficulty the following method was used:

Any strong cylindrical object such as a strong tin or porcelain or flat glass jar 11 cm. in diameter is taken and placed in the center of the glass plate. A moistened disc of filter paper under the jar will help to hold it in position. By means of the aluminum stick a circle is drawn on the glass plate around the jar. This procedure is not easy, for it is necessary to apply pressure and to traverse the same area with the pencil repeatedly. The surface of the glass must be damp, not wet; the best way of keeping it so is to breathe on it constantly. If the surface of the glass has been previously treated with a little sodium silicate, the procedure becomes easier. When properly done, the circle should give a silvery reflection, the line itself being almost 1 mm. wide. This line can be removed only with a strong NaOH solution.

If an acidulated solution of celloidin is now poured in the center of the circle and spread so as to overlap this line, the collodion will stick to it at the edges. At the same time, due to the presence of acid in solution, it will not adhere to the remaining surface of the glass, thus remaining stretched. Once the membrane is washed, a disc which can be removed from the glass without difficulty can be cut from the center. The aluminium line wears out by use and must be renewed from time to time. If the film still detaches from it, it means that too much acid has been added to the diluting mixture.

Sometimes difficulties are experienced in getting the celloidin to spread evenly when poured within the circle. This means that the glass is not clean.

If for any reason the addition of hydrochloric acid is not desirable, the best method of preventing the membrane from sticking to the glass is to cover the surface of the glass with a very thin film of gelatin. It is sufficient to smear the glass with cotton

wool soaked in 2 per cent gelatin and leave it to dry. The aluminium line must, however, be drawn afresh every time the glass has been coated with gelatin.

PREPARATION OF COLLODION MIXTURE

The ingredients are mixed fresh in the graduated test tubes in quantities sufficient for one disc. The ingredients which are intended to change the permeability of the membrane are first added, i.e., the acetic or formic ether, as the case may be. As very small quantities of these substances produce considerable changes in the membrane, they must be accurately measured by means of a 1 cc. pipette having 0.01 cc. divisions. The acidulated alcohol-ether mixture is then added up to the 9 cc. mark and well mixed. The stock solution is added last by pouring it in a fine stream into the middle of the liquid up to the 15 cc. mark. The tube is then corked with the cork stopper and vigorously shaken so as to produce a thorough mixture of the contents. A good deal of froth and numerous bubbles are produced. If now the cork is taken out very abruptly, with a jerk, an instantaneous vacuum is created above the collodion, and the bubbles rise quickly to the surface. That is why a sufficiently long and almost cylindrical cork must be used which acts as a piston. When all the bubbles have come to the surface and burst, the solution is ready to be poured on the glass plate.

MAKING THE FILM

Before mixing the collodion solution, the glass plate must be placed on the levelling table and levelled carefully. In order to mark each film for record purposes the number of the film is now written in reverse characters (as in "mirror writing") with an ordinary laboratory wax pencil on the surface of the glass. When the film becomes detached the number will adhere to the membrane. The celloidin mixture is now quickly poured into the middle of the circle. If the glass is clean and properly levelled the liquid distributes itself equally in all directions and is assisted to do so by means of the mouth of the test tube so that its edges finally overlap the aluminium circle to the extent of about 5

mm. If air bubbles appear, they can be easily removed by touching them with a metallic or glass rod. This must be done within the first minute of the liquid being poured on, for if done later it will alter the structure of the film. A wooden object or a piece of paper is not suitable for this purpose, as they only increase the number of bubbles. Small bubbles which may appear later are not of importance and the membrane should not be rejected if there are a few of these left. It is natural to think that a membrane containing bubbles will be more permeable than one without them, but experience shows the contrary to be the case as the following experiment proves.

Two membrane filters are mounted in a Zsigmondy funnel. The first of these, which has some bubbles in it, is of sufficient permeability to permit the partial passage of India ink particles. The second, which is placed below the first, can be of any permeability. Diluted India ink is now filtered through the membranes. The smallest particles of ink pass through the upper membrane and stain the lower one when the particles have passed through. Now, if the bubbles in the first membrane were more permeable than the other parts, we should expect to find black spots on the second membrane corresponding to the places occupied by the air bubbles in the first membrane. No such condition, however, is ever found for the following reasons: The air bubbles do not make a hole in the membrane. They are really small chambers covered on both sides with a very thin film. This film dries more quickly than the rest, and by so doing produces a less permeable membrane. Thus, at the site of a bubble the liquid has to pass through two membranes instead of one. The removal of bubbles is recommended only because they are unsightly.

At the moment the mixture of collodion is poured on the glass plate the stop-watch is started. A photographic darkroom timer is most convenient for this purpose, as it will signal the time when the membrane must be put into water.

The time taken in drying is most important. The permeability of the membrane depends upon the quantity of alcohol and ether present in the film at the time when the membrane is put into

water, and also upon the humidity of the air. This last factor is one which is not under our control in the ordinary laboratory. *Ceteris paribus*, however, the more humid the air is the more permeable is the membrane obtained. In my experience a membrane prepared when the humidity of the air is nearly 90 per cent is about twice as permeable as one prepared in dry weather when the humidity is 40 per cent. These variations in humidity are used by Zsigmondy and Bachmann in their patent method of preparing graduated membrane. In order to reduce discrepancies from this source to a minimum, the period of drying should be cut down as much as possible. The correct period is the time required for the collodion to set on the glass, i.e., the time necessary for the ether and other solvents to evaporate to a certain extent, but not the alcohol. The humidity of the air has practically no influence on the rapidity of evaporation of the solvents. The time required for the collodion to set is influenced mainly by the temperature of the air and by the amount of solvent used. The following equation, which has been evolved after numerous observations, probably gives the most accurate estimate of the time required:

$$T = K(30\text{-}t) + 7 + 2.5n$$

where T is the optimum time required to dry the membrane in minutes, t = the temperature (centigrade) of the air, n = the number of cubic centimeters of solvents other than ether-alcohol added, and K = a constant found experimentally for every batch of collodion. Usually K lies somewhere between 0.25 and 0.3. Thus, if the temperature of the air is 21°C., 0.5 cc. of acetic ether is added to the mixture, and K is 0.28, the optimum time of drying will be ten minutes forty-five seconds.

For practical purposes the equation can be plotted on a squared paper as a straight line.

The constant K is easily found experimentally. Celloidin of proper strength prepared from a new batch is poured as already indicated, and the stop-watch set in motion at the time the solution reaches the glass. The correct time to stop evaporation by immersion in water is reached when the sensation of a liquid

under the superficial film disappears and is determined by feeling the membrane lightly with a finger.[2]

Another formula which also gives satisfactory results, particularly for the higher temperatures (28°C. and upwards) is

$$T = \frac{210}{t} + 2.5n$$

where 210 is a constant found experimentally for the majority of batches. This method gives uncertain results with temperatures lower than 20°C.

When the membrane has set sufficiently, the glass plate is put into running water, in which it must remain until all the solvents are washed out. This process is complete when the water over the surface of the membrane spreads evenly and usually takes about fifteen to thirty minutes.

To prepare a filter for use, a Petri dish cover 91 to 93 mm. in diameter, is put over the middle of the film and a round disc is cut out of it with a knife. The knife must be very sharp, otherwise the edges of the disc will be ragged. The disc can be preserved either in 0.5 per cent formalin or in chloroform water. I prefer the former, as it is less volatile, but still disappears completely during sterilization. The discs are kept in round covered glass jars 14 cm. in diameter.

In addition to the single discs described, it is also possible to prepare a membrane from which several discs can be cut. Such a procedure has the great advantage of considerably shortening the time of preparation required for a number of membranes, and—what is more important—of obtaining several filters of the same permeability. For this purpose a square must be drawn on a large glass plate with the aluminium pencil. The larger the surface the wider must be the line in order to withstand the greater tension of the film. Alternately, a circle of some 30 cm. can be drawn making a celloidin disc 31 cm. in diameter. Seven membrane filters can be cut from a circle of this size. To get results with larger membranes comparable to those with single mem-

[2] Pierce in a recent paper advocates a better method; the film is sufficiently dry if its edge leaves a clean surface when lifted with a knife blade.

branes, the quantity of celloidin solution per unit of surface must naturally be the same. The quantity is easily calculated, knowing that, for the surface of a single disc of 113 sq. cm. area (12 cm. in diameter), 15 cc. of freshly made celloidin solution are used. As will be shown in my next paper, the freshly made mixture can be diluted with any convenient amount of ether without appreciably changing the permeability of the membrane. This dilution makes it easier to pour the celloidin solution over a large area. Even if all the conditions of preparation are apparently identical, however, a large membrane is eventually more permeable than a single membrane if prepared in a wet atmosphere, and less permeable if prepared in a dry one. This is due to the longer time required to dry larger membranes. The correct time of drying for large membranes is determined in the same way as that for the single standard membrane, K being found experimentally for each size of the covered surface.

Once the filter has been cut the remnants can be dried and used again in the same way as dry celloidin, as the solvents do not change its chemical composition. They must not be kept too long in the dry state however, as nitrocellulose changes to oxycellulose in the presence of air.

The amount of dexterity required in making large membranes is much greater than that required in the production of small ones. I, therefore, advise that their preparation should not be attempted until the technique required to produce the smaller filters has been thoroughly mastered.

STUDY ON COLLODION MEMBRANE FILTERS

PART II

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Received for publication June 22, 1932

MANIPULATIONS OF THE MEMBRANE FILTERS

In a previous communication[1] I gave the technique of preparation of collodion membranes. This paper is concerned with the use of these membranes and with the influence of different factors on their properties.

The flat collodion membranes can be used in two ways: (*a*) as a partition between two liquids, as in the twin tubes, described below; (*b*) as a filter in Zsigmondy's funnel.

The proposed twin tubes[2] serve the purpose of replacing collodion sacs by flat membranes, whose properties can be studied and changed according to the aims of the experiment. The tubes require only a small piece of membrane, and it is possible to cut such pieces out of a large one, producing in this way many membranes of almost identical properties. This enables experiments to be controlled adequately and to be repeated under identical conditions.

Twin tubes permit also the use of any other kind of membrane, e.g. parchment, bladder, amnion, etc.

Each pair of twin tubes consists of two tubes with a side opening at the enlarged lower part. The edges of those openings are ground and are large enough to hold tightly a piece of membrane between two rubber washers. The tubes are held together by two metal belts.

[1] Jour. Bact., **25**, 323.

[2] Manufactured by Les Establissements Leune, 28 bis rue du Cardinal Lemoine, Paris, France.

Combined in such a way, they give two compartments separated by the membrane, which can be filled with the same or

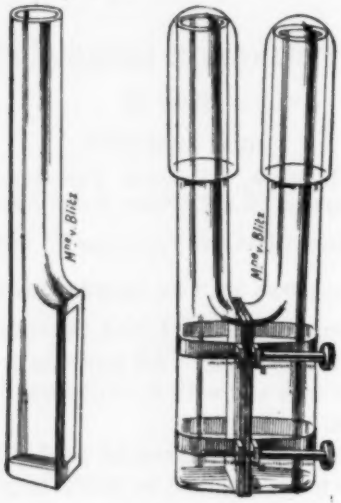

FIG. 1

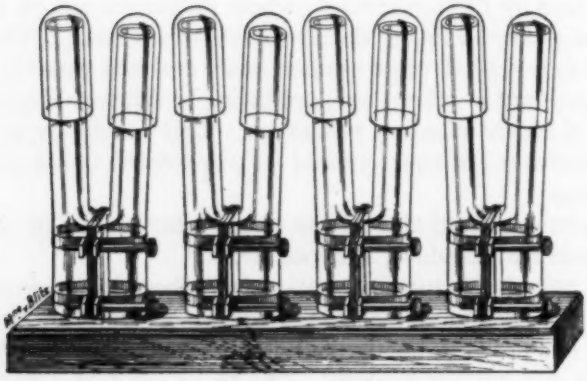

FIG. 2

different liquids and sterilized if necessary. The whole membrane is 27 by 70 mm., the working surface being 16 by 60 mm., that is

to say approximately 10 sq. cm. Each compartment holds 30 cc.

For bacteriological work the usual cotton wool plugs cannot be used, as one plug will catch fire when the other tube is opened and its mouth flamed. For this reason the tubes are provided with glass caps which are quite safe in protecting the medium from contamination.

The long neck of the tube is inclined in two directions: backwards and leftwards. This ensures more comfortable handling and at the same time prevents the liquid from being spilt out of one tube when manipulations are being carried out in the other, with both inclined.

Different grades of anaerobiosis can be obtained easily by covering the surface of the liquid with paraffine oil or by placing the whole apparatus in an anaerobic jar.

This feature of the tube promises a very large field of usefulness, which is at present impossible even to foresee. It appears that twin tubes might be of a considerable service in the following fields:

(*a*) In the study of ultraviruses (Protobes) and particularly the bacteriophage.

(*b*) In the study of filtrable forms of bacteria.

(*c*) In the study of symbiosis.

(*d*) In studies on synergism and the reciprocal influence of microörganisms.

(*e*) In the study of bacterial metabolism. If one tube is seeded with microörganisms, the products, if a sufficiently porous membrane is used, will pass into the other tube; the liquid can be taken out of that tube at any desired moment without interfering with the growth in the first tube.

(*f*) In the study of delicate microörganisms like *B. pneumosintes*. A piece of tissue can be placed in one compartment, the other being inoculated with the microörganisms (Gates' method).

(*g*) In the cultivation of protozoa. The protozoa can be cultivated in one compartment, the nutrient medium being changed regularly in the other. That will dispense with too frequent transfers of the organisms.

For the measurement of its permeability and for use as a filter the membrane is mounted in a Zsigmondy funnel.[3] This consists of an ordinary procelain funnel with a widened ground border, a concave or flat perforated disc to support the membrane and a bordered porcelain ring to hold the membrane and the liquid above it. Two aluminium rings tightened by means of three screws are employed to connect up the whole system and India-rubber rings are placed between each of the porcelain parts (fig. 3).

A thick filter paper disc, 7 cm. in diameter, is first placed on the perforated disc and the membrane laid on it. The paper disc is required to ensure that the whole working surface of the mem-

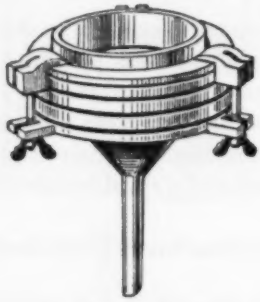

FIG. 3

brane is brought into play and not only the parts lying above the holes of the porcelain plate.

All parts of the apparatus must be dried with an absorbent rag, particularly the India-rubber rings, which must also be slightly powdered with french chalk, so as to prevent adhesion either to the membrane or to the porcelain during sterilization. The surface of the membrane is also cautiously dried with filter paper to prevent the talcum from being squeezed out while the various parts of the funnel are being clamped together.

If a concave supporting plate is used, the membrane needs special preparation to enable it to withstand the necessary distortion. It is first mounted in the ordinary way, all the parts of the funnel being tightly clamped together with the metal rings.

[3] Can be obtained from Membrane Filter, Fabrikweg 2, Gottingen (Germany).

Although sometimes recommended, the screws should *not* be slackened, as the edges of the membrane will tend to slip inwards while the membrane is adjusting itself to the concave surface and the joint between it and the porcelain plate will not have the required tightness. Once the membrane is in position, the funnel is mounted on a suction flask and a liberal amount, 20 to 30 cc., of alcohol is poured on it. This makes it much more elastic. A very slight negative pressure is now applied. This is best done with the mouth, the suction continuing very gradually until the membrane lies on the surface of the concave plate. The alcohol is now either poured off or allowed to filter through and is replaced by water which is also filtered through to wash out the alcohol. It must be remembered that alcohol treatment can only be applied to very porous membranes. The permeability of less permeable membranes changes considerably under the influence of alcohol.

The best way to ensure satisfactory working is to mount the funnel and the test tube which is to receive the filtered liquid, before sterilization. This is done as follows:

An India-rubber stopper, not more than 30 to 35 mm. in length, whose diameter corresponds to the neck of the suction flask to be used, is bored with a hole of 9 mm. The upper part of the boring is suitably enlarged to allow of the conical part of the funnel being inserted very deeply into it, thus leaving free more of the tube part of the funnel. A second stopper 20 to 25 mm. long and about 24 mm. in diameter is perforated with two holes—one 7.8 mm. and the other 1 mm. broad. A cuneiform piece from the upper edge of the smaller stopper is cut out, so as to make a side opening for the smaller hole. A capillary glass tube, one of whose ends has been ground like the point of a hypodermic needle, is now inserted into the small hole and the stopper in its turn adjusted to the stem of the funnel below the larger stopper. A test tube is then fitted to the smaller stopper (fig. 4). Another method is to replace the small India-rubber stopper with a non-absorbent cotton wool plug, which must be wound round the funnel tube very tightly. Precautions must be taken to prevent this plug from being moistened during sterilization and filtration. After

filtration the funnel is taken off and the test tube removed and plugged with sterile cotton wool.

An air filter inserted so as to filter the air returning to the flask prevents contamination of the filtered solution.

FIG. 4. THE MOUNTED ZSIGMONDY'S FUNNELS

1 shows the section of india-rubber stoppers; *2* shows how cotton-wool pad is fixed with perforated disc and two springs.

During sterilization of the apparatus the membrane is apt to distend and become waved, so that when it is exposed later to negative pressure, it is liable to get folded and to break. Where a concave plate is used, the film may become unduly stretched and torn if the suction is not applied very cautiously.

To prevent this a strongly rolled disc of cotton wool 2 to 3 cm.

thick and of the same diameter as the open part of the membrane is placed on the membrane. This is thoroughly moistened and covered with a perforated porcelain disc 6 cm. wide, one that is used to support the cotton wool in an ordinary funnel. The disc is then strongly pressed down and held tight by two springs. To note the number and permeability of the membrane a small ivory key label is attached and necessary notes written with an ordinary pencil. These labels stand the sterilization well.

The whole apparatus can now be safely sterilized in the autoclave at 110°C. for fifteen to twenty minutes. Sterilization at higher temperature affects both the membrane and the India-rubber parts.

MEASUREMENT OF PERMEABILITY

When estimating the permeability of a filter, it must be remembered that the meshwork of the membrane is not regular, but consists of a number of pores of *varying* and unknown size. In filtration work only the size of the largest pores is of importance, for it is by these alone that the retaining capacity of any filter is judged. Unfortunately, there is no simple method of measuring the size of these. The following methods have been suggested for this purpose:

1. The use of different colloidal suspensions containing particles of known size. This method perhaps would give reliable results if phenomena which occur during the process of "filtration" were known. Up to the present, no series of graduated suspensions each of which contains particles of known size, electrical charge and absorptive power has yet been satisfactorily prepared.

2. Bechhold's method implies the determination of the pressure at which a wet membrane allows air to pass through. This method is too complicated for routine use, as it requires special apparatus and can only measure narrow ranges of permeability.

3. Guérout's method is based "on the assumption that the membrane acts like a bundle of capillary tubes having a length equal to the thickness of the membrane and that the law of Poiseuille applies to the flow of the water through such short and narrow tubes" (Hitchcock[4]). This method appears to be more

[4] Jour. Gen. Physiol., 1926, **9**, 755.

practical, but is still too complicated for routine use. Moreover, it determines the mean diameter of all the pores and not the diameter of the largest ones.

Recourse must, therefore, be had to a method which has the merit of practical convenience and by which we can compare different series of membranes. Determination of the actual size of the biggest pores or of their mean diameter can be used as a control of the simplified method described below.

4. Experience and comparative measurements of the pores by one of the methods mentioned above show that the difference in the rate of the flow of the water through a series of membranes similarly prepared depends mainly on the difference in the size of their pores. If the size of the pores is reduced, however, the number per square unit may increase and the ratio between size and rapidity of filtration will be altered. Nevertheless, for practical purposes we can accept the fact that the less the rate of the flow the smaller are the pores. If, therefore, we determine the time necessary for a definite amount of water to pass through a membrane of definite size under a fixed pressure, we can then compare membranes of our series and by arranging them in order of the time data obtained, we will at the same time arrange them according to the size of their pores. Such a determination is fairly satisfactory for routine work. It permits of membranes being divided into convenient groups according to their permeability. It is thus possible to study the properties of these groups and select the particular membrane suitable to the requirements of the work in hand, and by using the same technique in their preparation produce others which are more or less identical.

This method is used for numbering the membranes prepared by Zsigmondy and Bachman's patent (D. R. P., Nos. 329, 117 and 329, 060) and put on the market by Messrs. Membrane Filter, Göttingen. These membranes bear the number of seconds necessary for 100 cc. of water to pass through 100 sq. cm. of membrane under a pressure of one atmosphere. As my membranes are prepared by a different method and, as consequently, the numerical data of the two are not comparable, I have found it advantageous to change the pressure required to 60 cm. of Hg. This

pressure is sufficient and is easily obtained by means of an ordinary suction pump.

In my method, measurements are made by means of a very simple device which I call a "Permeometer," and which has proved to be very reliable and fairly exact. It operates on the same principle already described for measuring the permeability of collodion sacs. The apparatus consists of a part of a burette or pipette mounted on a large India-rubber stopper corresponding to the opening of a Zsigmondy's funnel. This is mounted on the funnel and the whole is fitted to a suction flask (fig. 5). The funnel and burette are filled with water until the level rises above the highest mark. When a negative pressure of 60 cm. Hg is applied, the water level in the burette begins to fall. When it drops to the upper mark on the burette the stopwatch is set going. The time required for the number of cubic centimeters of water equal to the number of square centimeters of the working surface of the membrane to filter through, is determined. Thus, if the surface of the membrane is 35 sq. cm. the number of seconds required for 35 cc. to pass through the membrane should be noted.

To shorten the operation, burettes or pipettes of three different sizes are used and the total amount of water measured varies according to the permeability of the membrane. Thus, for a permeable membrane (10 to 100 seconds), a large burette of some 45 cc. capacity is employed and the whole quantity of liquid, i.e., 35 cc. is allowed to pass through. For less permeable membranes (100 to 1000 seconds) a 5 cc. pipette divided into tenths is used and 3.5 cc. of water are measured, while for very dense membranes (1000 to 4000 seconds) a 1 cc. pipette divided into hundredths is used to measure only 0.35 cc. of water.

In this last case, it is impossible to fill the funnel through the 1 cc. pipette. For this purpose, a small separating funnel with a long stem is inserted through the stopper along with the pipette and so arranged that the level of water in the former can reach above the top mark on the pipette. The apparatus is filled through the funnel, the air escaping up the pipette, until the latter is filled with water. When the stopcock of the funnel is closed and pressure applied, the water level in the pipette begins

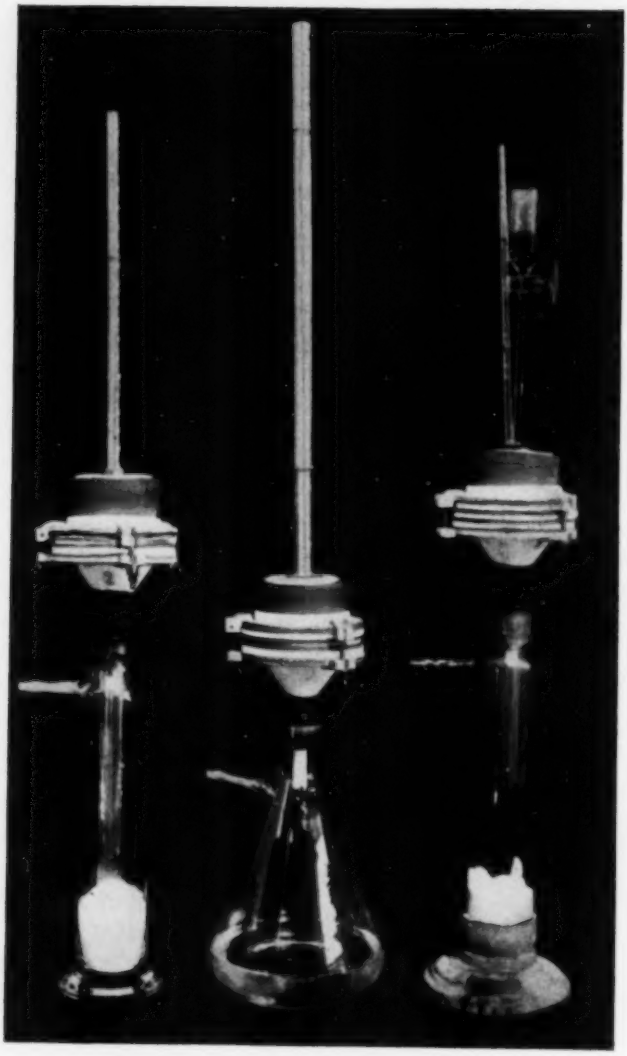

FIG. 5. PERMEOMETERS FOR MEMBRANE FILTERS

to fall (fig. 5, right). Such measurements give fairly accurate results. The mean of several measurements should be taken. This mean number of seconds necessary for the required amount of water to pass through the membrane gives the "number of permeability." Practice shows that only a relative accuracy is all that is required. Differences of one second should be noted only with membranes whose number of permeability is less than 35 seconds; up to 150 seconds an approximation of 5 seconds is sufficient; with membranes of still lower permeability 50 seconds approximation will be adequate. When standardized, the membranes are either mounted for sterilization or put into 0.5 per cent formalin until required.

A closer study of the properties of membranes can be carried out by means of colloidal suspensions mentioned above. As the properties of such solutions depend on many different factors, it is advisable that the test solutions should be prepared specially to suit the particular line of investigation to be undertaken. It must be remembered that true filtration can only take place if the colloidal solution has a negative charge and if the substance used is not adsorbed by the membrane.

The membrane itself being electronegative, the size of the pores alone will determine the passage or retention of suspensions made up of particles carrying a negative charge. On the other hand suspensions containing positively charged particles will be retained in most cases quite independently of the relative dimensions of the particles and pores, because the phenomenon of coagulation and adsorption will take place.

In the case of electronegative suspensions, however, the phenomenon of adsorption introduces a very serious complication. Many electrically negative suspensions with particles of small size will not pass through a large pored and negatively charged membrane, but will be adsorbed by it. Thus, for instance, a solution of congo red, a negative colloid, will not pass through a very porous membrane until saturation of the membrane has occurred.

For coloured solutions, "capillarisation" is the best method of

determining whether or not the solution is adsorbed by the membrane. A strip of *dry* permeable membrane is dipped into the solution. If the dye diffuses up almost as rapidly as the water, it is not adsorbed (e.g., acid fuchsin); if, however, the pure water diffuses up, the dye is strongly adsorbed (congo red). A still better method is the observation of the widening of a drop of coloured solution placed on a piece of dried membrane.

INFLUENCE OF INGREDIENTS ON THE PROPERTIES OF MEMBRANES

Ether. Ethylic ether serves only as a solvent and a diluent. It must be pure and, especially, free from acid. The amount of ether added has only a very slight influence on the resulting permeability of the membrane, provided that the same amount of celloidin and of the other ingredients be used per unit of surface. This property of ether is of advantage when it is found necessary to dilute the celloidin solution to make it easier to spread on the surface of the glass. The time of drying is only slightly increased under these conditions and the permeability of the membrane will be slightly increased or decreased due to the longer exposure to the dry or humid air respectively.

Alcohol. The addition of alcohol makes the membrane more permeable. At the same time it makes it brittle and more difficult to handle. For that reason I prefer to keep the quantity of alcohol used as low as possible.

Ether-alcohol mixture. This acts as a solvent and a diluent, nitrocellulose not being soluble in either of the two separately. Used as a diluent it changes the membrane in two ways: it reduces the celloidin content, and relatively increases the quantity of alcohol, thus producing a more permeable and brittle membrane.

Used as a solvent, the proportions alcohol 25 and ether 75 parts were chosen as containing the smallest quantity of alcohol required to dissolve the celloidin while at the same time giving a membrane of sufficient strength.

Compared with other solvents, the alcohol-ether mixture has the following advantages:

1. The film is not left to dry completely, as is the case, for instance, in Bachmann and Zsigmondy's process, but is put in water

immediately after the ether has evaporated and the collodion has set in the alcohol which remains. As a result of this treatment the process takes less time and the membrane is much less influenced by the humidity of the air. For instance, Zsigmondy and Bachmann in describing their patent process give an example where membranes were prepared with a solution of celloidin in a mixture of acetone-acetic acid, and dried in atmospheres of varying humidity. The same solution gave a membrane of 50 seconds in a humid atmosphere and a nonpermeable one in a dry one. To determine the influence of humidity on my membranes I placed the films under a bell jar, in an air current which was either dried by calcium chloride or moistened by passing it through water. The membrane which set in damp air was only twice as permeable as the other one.

2. Experiments seem to indicate that membranes of similar permeability and containing the same amount of celloidin per unit, but prepared with different solvents, have pores of very different dimensions. Thus, in my hands a 20 seconds- or even a 25 seconds- membrane prepared with a 6 per cent solution of celloidin in acetone-acetic acid often let bacteria pass. On the other hand, bacteria were always retained by a 15 seconds-membrane made with an alcohol-ether solution of similar concentration (with addition of acetic ether). This means that an "alcohol-ether" membrane has a greater number of smaller pores per unit of the surface than a membrane made with acetone-acetic acid and consequently a similar degree of filtration can be obtained with the former in a shorter time.

Celloidin. Increasing the quantity of celloidin per unit of surface results in a more solid and less permeable membrane, not because the pores are smaller but because their arrangement is more complex.

A mixture of 6 cc. of 10 per cent celloidin with 9 cc. of 25:125 alcohol-ether for a surface of 12 cm. in diameter (113 sq. cm.) is recommended as the one giving the best film. Such a film is firm enough to permit easy handling and yet thin enough to obviate excessive adsorption. For special purposes the quantity of celloidin per unit of the surface can be reduced more than one-

half. In this case, however, I would recommend that the mixture be made up to the total volume of 15 cc. with ether and not with alcohol-ether alone.

The results obtained with different brands of nitrocellulose have scarcely been investigated, because of the difficulty of obtaining stable products on the market. (Nitrocellulose cannot be sent by post.)

Nitrocellulose even of the same make, however, often gives different results. Thus, membranes prepared from different stock solutions made from the same brand of celloidin show a different permeability. In my hands the variations have been as much as 25 per cent. Some batches give a very even film, while in others the "net" design is very pronounced. Some batches again are much more or considerably less sensitive to the addition of the different ingredients than others. It follows therefore that every stock solution must first be "titrated" by measuring one or two membranes prepared with standard dilutions, e.g., 6 cc. stock solution with 9 cc. alcohol-ether mixture.

Acetic ether. If acetic ether is added to a solution of celloidin it greatly increases the permeability of the membrane. A very small quantity—0.1 cc. for 15 cc. of celloidin solution—is sufficient to produce this effect. Table 1 gives an example of the variations produced with one of the batches.

It is obvious that celloidin solutions are very sensitive to the addition of acetic ether, as the addition of 0.1 cc. produces a change of about 50 per cent in the permeability of the resulting membrane. The addition of the acetic ether in smaller quantities, such as 0.01 cc., gives irregular results and is, therefore, of little use.

Formic ether. It has the same action as acetic ether, but has the advantage that the change of permeability is much more gradual. Used in combinations with acetic ether it helps to produce a series of membrane filters with a very gradual rise in permeability. Table 2 gives an example of this.

Amylic alcohol. In a previous publication,[5] I pointed out that amylic alcohol has a reverse action to the acetic ether, i.e., its

[5] C. R. Soc. Biol., 1924, **92**, 362.

addition reduces the permeability of the membrane. This was true for the particular batches of colloidin and amylic alcohol used at that time. Further tests have shown, however, that the action of amylic alcohol is very irregular and can not be depended on. As a detailed chemical study of the cause of these irregularities could not be carried out, its use was discontinued. A more reliable substitute for amylic alcohol, by means of which the permeability of the membrane can be reduced, has not yet been found. If a series of membranes is required in which the permeability has to be very low, say, 2000 to 4000 seconds, it is necessary to

TABLE 1

STOCK SOLUTION	ALCOHOL-ETHER, 25:125	ACETIC ETHER	PERMEABILITY
cc.	*cc.*	*cc.*	*seconds*
6	9	0.0	1,100
6	8.9	0.1	600
6	8.8	0.2	300
6	8.7	0.3	180
6	8.6	0.4	125
6	8.5	0.5	50
6	8.4	0.6	35
6	8.3	0.7	25
6	8.2	0.8	15
6	8.1	0.9	10
6	8.0	1.0	7

$t° = 26.8°C$. Humidity of the air = 65 per cent.

prepare them in dry weather (35 to 40 per cent humidity) and to increase the constant K in the drying formula some 25 per cent.

Humidity of the air and its temperature. As pointed out by Zsigmondy and Bachmann (Deutsches Reichspatentamt, Patentsschrifte Nos. 329,060 and 329,117, November, 1920) permeability varies considerably with the humidity and temperature of the air; e.g., the hotter and drier the air, the less permeable will be the membrane obtained. It is almost certain that the divergent results obtained by different observers were due to their preparing the membranes under different humidities and temperatures of air. Thus, a membrane prepared in winter in a cold and wet atmosphere will have a permeability of, say, 400 to 600

seconds while precisely the same technique will produce one of 1000 to 1200 seconds during dry weather in summer.

To overcome this difficulty, it is advisable, as a preliminary to the preparation of membranes of any particular permeability, to prepare test membranes: one without any additional ingredients and one with large amount, say, 0.8 to 1 cc. of acetic ether. After practice is gained, the amount of acetic or formic ether to

TABLE 2

STOCK CELLOIDIN	ALCOHOL-ETHER, 1:5	ACETIC ETHER	FORMIC ETHER	PERMEABILITY
cc.	*cc.*	*cc.*	*cc.*	*seconds*
6	9	0	0	1,000
6	8.9	0	0.1	900
6	8.8	0	0.2	600
6	8.7	0	0.3	420
6	8.6	0	0.4	280
ANOTHER SERIES				
6	8.9	0.1	0	350
6	8.8	0.1	0.1	210
6	8.7	0.1	0.2	160
6	8.6	0.1	0.3	90
6	8.8	0.2	0	90
6	8.7	0.2	0.1	75
6	8.6	0.2	0.2	60

TABLE 3

	PERMEABILITY (SECONDS)									
Before sterilization	1,100	790	105	60	40	35	25	15	12	12
After sterilization	1,090	600	107	75	53	42	36	21	28	20

produce a membrane filter of any particular consistency can be easily estimated.

Sterilization in the autoclave affects appreciably the permeability of porous membranes only (table 3).

The second sterilization affects permeability but slightly.

Nutrient media. After prolonged (a fortnight or more) contact with such liquids as nutrient broth, membranes become somewhat brittle. This action is accelerated if the membrane is sterilized

in contact with an organic fluid. For this reason, it is essential that a membrane before subsequent sterilization should be washed thoroughly as soon as possible after filtration.

THE USE OF MEMBRANE FILTERS

Membrane filters have almost entirely replaced other filtering appliances in my laboratory because their manipulation is easier and the results obtained with them are more consistent.

As the chief property of collodion membranes depends on the fact that when a suspension of electronegative corpuscles (bacteria, etc.) which is not adsorbed by the membrane is filtered, the corpuscles are retained by the *size* of the pores and not because of the intricacy of channels (as occurs in porcelain filters) it follows that the microörganisms can never *grow* through the membrane, and further that the electro-negative corpuscles, if retained, lie on the surface of the membrane only and not in the pore channels. Collodion filters thus have two great advantages from the technical point of view: (*a*) the sediment on the membrane can be collected with very little loss, and (*b*) the filters can be very easily cleaned and thus can recover a great part of their filtering capacity.

On account of these advantages, I have used membrane filters in bacteriophage work and for collecting bacteria from large quantities of water when searching for *B. typhosus;* and have used them also for preparing Besredka's antiviruses where large quantities of bacterial suspension are filtered.

Very small quantities of liquid can also be filtered through the membrane filter with very little loss. In my current work on the bacteriophage I always use one or two 10 cc. volumes of broth. With such small quantities it is not necessary to clear the liquid by preliminary filtration through infusorial earth, as is done when using porcelain filters. The process of filtration goes quickly and easily; with a new membrane filter of 25 seconds some 20 cc. of medium heavily charged with a secondary growth of bacteria can be filtered in about one minute. A membrane filter which has been used repeatedly will take a few minutes. With a particularly heavy growth or with mucous liquids (pyocyaneus cultures)

the sediment collecting on the membrane has to be removed with a fine water-colour brush.

In working with membrane filters, instead of mineral ones (Chamberland, Berkefeld, etc.) we have the advantage of dealing with a factor which can be more easily studied. In mineral filters the intricacy of the channels and adsorptive power of the filter play the main rôle. In changing the porosity of a candle filter we introduce unknown factors, whereas the properties of the membrane filters can be much more closely controlled. Many fields of biological research would gain much in exactness and certitude if standardised membrane filters were used for purposes of filtration. Thus, in using the membrane filters with pores of known size it would almost certainly be possible to *divide* the ultramicroscopic world into convenient groups and improve on the present vague notions held as to "filtrable forms of bacteria," "filtrable viruses," etc.

Considerable experience in the preparation of membrane filters and in the study of their properties has convinced me that, under proper conditions of experimental work, sufficiently accurate laws governing the properties of these membranes and the methods to be employed in their preparation can be obtained. A room specially adapted for the purpose would, however, be required to carry out the necessary experiments. This room would have to be well ventilated, but without obvious air currents and, most important, it should be possible to change the temperature and humidity of the air at will. Working under such conditions, I am sure that it would be possible to discover exact rules which would enable anyone to prepare membrane filters of the required permeability under any conditions.

The aim of this publication is to attract attention to the advantages of membrane filters and to share my technical experience with other workers who may find opportunities for a more exact study of the question.

SUMMARY

The technique of the preparation of collodion membrane filters is described and is considered easier and capable of giving more

constant, comparable and exact results than many other methods. Such filters enable the results obtained to be standardised and minimize the effect of uncontrollable factors.

Collodion membrane filters have many advantages over other types of filters.

Note. This paper was completed before I read Pierce's paper on the same subject.[6] Our techniques differ considerably. His drying cameras are ingenious, but not easily made available in all laboratories. In my opinion a combination of his and my technique would give better results than each separately. It would be particularly desirable to introduce a large drying chamber of the A type, so that my technique for preparation of membrane filters could be applied in it under more stable conditions.

[6] Jour. Biol. Chem., 1927, **75**, 795.

SECONDARY COLONIES OF BACTERIA WITH SPECIAL REFERENCE TO BACILLUS MYCOIDES

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Received for publication June 22, 1932

INTRODUCTION

Although the phases of growth which occur in a primary colony of bacteria are well known, our knowledge of the origin and significance of secondary colonies is incomplete and in many respects unsatisfactory. It has long been known that, under certain conditions, the colonies of many species of bacteria are capable of resuming growth after a more or less extended period of senescence. This secondary phase of growth may be of short duration, resulting in the formation of minute papillae, or it may be so prolonged as to result in the formation of well developed daughter colonies.

Various explanations have been offered to account for this phenomenon, but there are still differences of opinion both as to its cause and its significance. The literature of the subject has been reviewed by other writers, especially Dobell (1912), Penfold (1911), Hadley (1927), Arkwright (1930) and Stewart (1927). Some brief references must be made at times to the experiments and conclusions of others, but no necessity exists for a full review of the rather extensive literature.

Secondary colonies have been reported for many species belonging to various genera, but the coliform bacteria have received more thorough study by experimental methods than other groups. With a single exception, the spore-forming species do not appear to have been very thoroughly studied.

The first experimental study of this troublesome problem appears to have been that of Preisz (1904). He noted that old cultures of some, but not all, strains of *B. anthracis* became so

studded with papillae and secondary growth as to resemble a mixed culture. Since secondary colonies were never observed in asporogenic races but developed in cultures of spore forming strains heated to 65°C. for one hour, he concluded that they owed their origin to germination and subsequent growth of spores within the primary colony. He noted the occurrence of similar colonies in cultures of *Vib. cholerae* and *B. diphtheriae*, which he attributed to the growth of cells with greater vitality and power of resistance to unfavorable environment. This early work of Preisz has been frequently quoted and appears to have been generally accepted, but evidence of confirmation for *B. anthracis* or other spore-forming species is lacking. On the other hand, there is much evidence that important factors are involved which he failed to recognize. The conclusions which he reached are, therefore, subject to doubt until fully confirmed.

Other spore-forming species which have been studied briefly are *B. subtilis* by Soule (1928) and *B. mycoides* by Lewis (1932). No attempt was made in either of these cases to determine the factors involved in the origin of such colonies, but experiments were performed in each case to determine whether or not the secondary colonies were due to dissociation. Subcultures from the smooth variety of *B. subtilis* were about equally divided between the two forms, while all strains derived from the rough colonies were of the rough variety. Subcultures from *B. mycoides* could not be distinguished from the parent strain.

The most important contribution to our knowledge of this subject was made by Neisser (1906) who first discovered the now well-known type which, because of its behavior on lactose media, is known as *B. coli-mutabile*. This organism does not ferment lactose, but gives rise to a subrace which has this capacity. This characteristic may be acquired in broth containing lactose or by papillation of colonies on lactose agar. The subrace ferments lactose and does not again produce secondary colonies. Reversion to the non-fermenting type occurs, in some instances, while in others very minute papillae have been observed on agar containing lactose. Since these fail to develop into true secondary colonies, their origin is doubtless due to some other cause.

This important pioneer discovery has been completely confirmed by Massini (1907), Burk (1908), Reiner Müller (1909), Burri (1910), Baerthlein (1911, 1912), Mellon (1925), Stewart (1927) and Kennedy, Cummings and Morrow (1932). The same principle has been shown to apply to many species besides *B. coli-mutabile*, and various sugars or alcohols may cause the phenomenon.

Penfold (1911) showed that *B. typhi* forms secondary colonies on agar containing dulcitol and that subcultures are enhanced in power to ferment this compound. Similar results were obtained by Bernhardt and Markoff (1912) for a strain of *B. dysenteriae* Flexner when cultivated on maltose. Reiner Müller (1912), worked with three strains of coliform bacteria which could not ferment arabinose but which formed papillated colonies on arabinose agar. Subcultures from the papillae still failed to ferment the compound, but grew more vigorously in its presence. He reported similar results with *B. typhi* on agar containing rhamnose. When cultivated on raffinose agar, *B. paratyphosus* B produced secondary colonies with enhanced capacity for fermentation.

Additional examples might be cited, but could add nothing of value, since the principle appears to be the same in all cases. Variation in presence of a specific sugar or alcohol with formation of secondary colonies which show newly acquired or enhanced capacity for fermentation of the specific compound and failure to produce secondary colonies has been shown in each case. The secondary phase of growth appears to be due, therefore, to beneficial variation.

Various other theories have been advanced to account for the development of this secondary phase of growth in colonies but, aside from the work of Preisz and that of the authors previously mentioned, there is not much evidence of experimental investigation. In most, if not all, of these cases the explanations are rather vague and with no foundation other than theoretical reasoning based on uncertain hypotheses.

Because *B. mycoides* was previously known to form secondary colonies under some conditions, it appeared to be a suitable

species for investigation. The present study is concerned specifically with an attempt to discover whether or not the secondary colonies arise in this species from spores or from vegetative cells, and whether the principle of variation in response to carbon compounds applies.

EXPERIMENTAL

The strains of *B. mycoides* employed in the experiments included the four strains (A, B, C and American Type Culture Number 80) previously studied as to dissociation and life cycle, Lewis (1932). Two variants of strain C and fourteen additional strains isolated from soil, air and water were also included.

The first experiment was performed to determine the extent of secondary colony formation on standard beef-extract peptone agar. Cultures consisting of giant colonies on the surface of agar in petri plates were held at room temperature for a period of ten days, protected against desiccation. At the end of this period strain C, previously known to form such colonies, its two variants numbers 22 and 94 and one additional strain, A2, indistinguishable from C, were positive, while all of the remainder were negative. The cultures were held for a total period of sixty days, but with no change in the results. It thus appears that this medium is unsatisfactory for the phenomenon or that it does not occur in a great majority of the strains.

Attention was next directed at the effect of specific sugars and alcohols. *B. mycoides* is generally recorded as producing acid from glucose, maltose, glycerol and sucrose, but not from lactose or mannitol. The several strains were cultivated as giant colonies on the surface of standard nutrient agar containing 2.0 per cent. respectively, of each of the above carbon compounds and the proper amount of phenol-red indicator. The plates were inspected daily for a period of ten days and at frequent intervals throughout the incubation period. The fermentation reactions which were to be expected occurred in all cases except sucrose. There was no formation of acid in the case of lactose or mannitol while all strains fermented glucose, maltose and glycerol promptly.

Differences in capacity to ferment sucrose were evident within

twelve hours. Six strains produced sufficient acid to cause complete reversal to the full acid color of the indicator during this period, while all others became more strongly alkaline, and remained so indefinitely. The acid strains eventually reverted to the alkaline reaction but only after a period of about two weeks.

Since simultaneous formation of acid and alkali or failure to produce acid under aerobic conditions might cause the results obtained for the non-acid strains, these were studied further in test tube cultures. The medium consisted of a 1.0 per cent peptone solution containing 2.0 per cent sucrose plus phenol-red indicator. Cultures were prepared in both liquid medium and in deep tubes of agar.

The liquid cultures of all strains showed some slight change toward the acid side of neutrality within twenty-four hours, but in no case was the full acid color reached except on prolonged incubation. Four strains reverted to the alkaline reaction within forty-eight hours, with no subsequent reversal of reaction to acid. The remaining ten strains reached the full acid color in periods varying from five to fifteen days, after which no reversal occurred.

The cultures in agar tubes became alkaline at the surface in a zone extending downward to a depth of about 1 or 2 cm., but in all cases the deeper layers became acid within twenty-four to forty-eight hours. This shows that these strains do not attack sucrose, or at least not vigorously, in the presence of oxygen but are capable of sucrose fermentation from the beginning under conditions of partial anaerobiosis. If any acid is formed at the surface it is masked by the more vigorous formation of alkali.

The slow fermenting strains of *B. mycoides* are thus seen to vary toward sucrose in much the same manner as do those of *B. typhi* to dulcitol, as shown by Penfold (1911), but in a somewhat different manner from the reaction of *B. coli-mutabile* to lactose. It remains to be shown now that this similarity in fermentation extends also to the matter of secondary colony formation.

Results on agar plate cultures are shown for secondary colony formation in table 1. All of the slow fermenting strains ex-

cept A_2, C and its two variants, 22 and 94, formed secondary colonies on sucrose, but not on any of the other carbon com-

TABLE 1

The effect of culture media containing 2.0 per cent agar and various nutrients on the formation of secondary colonies by some strains of B. mycoides

NUMBER OF STRAIN	COMPOSITION OF MEDIUM											
	Beef extract, 0.3 per cent Peptone, 0.5 per cent	Beef extract, 0.3 per cent Peptone, 0.5 per cent Sucrose, 2.0 per cent	Beef extract, 1.0 per cent	Aminoids of beef, 1.0 per cent	Bacto peptone, 1.0 per cent	Bacto tryptophane broth, 1.0 per cent	Bacto peptone, 1.0 per cent Lactose, 2.0 per cent	Casein digest	Bacto peptone, 1.0 per cent Glucose, 2.0 per cent	Bacto peptone, 1.0 per cent Sucrose, 2.0 per cent	Bacto peptone, 1.0 per cent Sodium acetate, 0.2 per cent	Jenner's peptone, 1.0 per cent
A.T.C.	−	+	−	−	−	−	−	−	−	+	−	−
A	−	−	−	−	+	+	+	+	−	−	+	−
B	−	+	−	−	−	−	−	−	−	+	−	−
C	+	−	−	−	+	+	+	+	−	−	+	−
A2	+	−	−	−	+	+	+	+	−	−	+	−
A6	−	−	−	−	+	+	+	+	−	−	+	−
22	+	−	−	−	+	+	+	+	−	−	+	−
23	−	−	−	−	+	+	+	+	−	−	+	−
94	+	−	−	−	+	+	+	+	−	−	+	−
402	−	−	−	−	+	+	+	+	−	−	+	−
405	−	−	−	−	+	+	+	+	−	−	+	−
407	−	−	−	−	+	+	+	+	−	−	+	−
410	−	+	−	−	−	−	−	−	−	+	−	−
416	−	+	−	−	−	−	−	−	−	+	−	−
417	−	+	−	−	+	+	+	+	−	+	+	−
419	−	+	−	−	−	−	−	−	−	+	−	−
420	−	+	−	−	−	−	−	−	−	+	−	−
421	−	+	−	−	+	+	+	+	−	+	+	−
422	−	+	−	−	+	+	+	+	−	+	+	−
424	−	+	−	−	−	−	−	−	−	+	−	−

+ = Secondary colonies. − = No secondary colonies.

Strains A, A6, 23, 402, 405, 407 caused prompt fermentation of sucrose.

Strains C, 22, 94 and A2 gave final alkaline reaction in sucrose peptone water.

Strains A.T.C., B, 410, 416, 417, 419, 420, 421, 422 and 424 caused delayed fermentation of sucrose with final acid reaction.

pounds tested. None of the rapid fermenters (A6, 17, 23, 402, 405, 407) produced secondary colonies on any of the media. It

is a point of considerable interest that the slow fermenters which failed to form colonies of secondary origin on sucrose agar had been previously shown to be the only positive strains on plain agar. An explanation for this will be offered in connection with additional experiments. In regard to secondary colony formation, the three groups of strains of *B. mycoides* react to sucrose in a manner almost identical with that of colon bacteria to lactose. The acid strains are comparable to *B. coli* which ferments the specific compound promptly but does not form secondary colonies. The strains which cause delayed fermentation are similar to *B. coli-mutabile*, while the four strains which fail to cause delayed fermentation or produce secondary colonies are more like *B. paracoli*.

A point of considerable interest was observed in the correlation between right and left hand spiral symmetry and sucrose fermentation. Prompt fermentation correlates, in general, with left hand spiral growth (strains A6, 17, 23, 402, 405, 407), while delayed fermentation correlates with right hand turning of the threads (strains A.T.C., A2, 22, 24, 62, 94, 410, 416, 419, 420, 424). The correlation was not quite perfect, since three strains (417, 421, 422) with left hand spirals were classed as slow fermenters and produced secondary colonies. Without exception the right hand strains proved to be unable to cause prompt fermentation of sucrose. It is no doubt true that the collection contained many duplicates which could not be distinguished from each other, but there were some strains in each group which could be readily distinguished by appearances of the colony and growth rate. Strains A2 and C differed from all other members of the right hand group by forming much longer and less robust threads. They differed also in not being capable of adaptation to sucrose, which was also true of the two variants from C, numbers 22 and 94. Strain 402 resembled other members of its group, but the growth on plain agar was much less vigorous and the colonies soon became scarcely visible as a film of glassy threads. When grown on sucrose agar, it could not be distinguished from other strains. It appeared to suffer from carbon deficiency in the absence of an added source. The sec-

ondary colonies appear rather promptly in all strains capable of forming them. The A.T.C. strain begins to show very minute papillae by the sixth day, while other strains require a somewhat longer period. In all cases where papillation had not occurred by the fifteenth day the result was negative at the end of two months.

The first papillae are invariably located on the oldest portion of the colony. For the purpose of examining the progressive development of the parent colony and the secondaries, the A.T.C. strain was found to be most satisfactory. It spreads much more slowly, requiring a period of about two weeks to cover the surface of the plate. The papillae likewise develop slowly and do not reach mature size until about the fifth day after they become visible. At maturity they are remarkably uniform in size, shape, and consistency. The maximum size is about 2 mm. in diameter. They are much whiter than the original threads of the parent colony and contrast sharply with the bluish background. Figures 4, 5, 6 show various aspects of this strain.

At the end of about three weeks the whole surface of the plate is thickly studded with secondary colonies. The parent colony as a whole grows but little, if any, better than on plain agar, and there is never any change in the size or vigor of filaments as they spread out from the line of inoculation. This indicates that only certain cells are capable of variation toward the sugar and that there is no mass transformation, as has been claimed by some previous workers.

Subcultures are easily obtained, since the colonies are relatively large and stand out well above the background of threads. Dilution plates prepared from a suspension of a bit of the colony invariably produce nothing but typical thread-like growths indistinguishable from the parent type. In one experiment forty subcultures were picked to sucrose agar slants from a plate prepared from a single secondary colony. These were subsequently transplanted to sucrose agar plates and tubes of peptone water containing sucrose. The results of this experiment showed that the secondary colony produces a race more active in fermentation of sucrose than the original, and not capable of again producing

secondary colonies. Thirty-five of the substrains belonged to this type, while five were like the original parent both as to slow acid formation and ability to form the secondary growth.

In this connection it should be pointed out that, with one possible exception, none of the original strains had been in previous contact with sucrose between the time of isolation and the performance of these experiments. The possibility that the active fermenters had acquired this habit through previous cultivation after the manner just described is thus excluded. A complete record of the A.T.C. strain was not obtained, but its behavior would indicate a non-sucrose history. This strain forms an abundance of secondary colonies when cultivated on sloped sucrose agar in test tubes, and it would appear that, after a few transplants made at long intervals, the original slow fermenting type might easily be completely lost.

Subraces were established from secondary colonies of each of the ten strains which produced them. These races not only fermented sucrose more rapidly than the originals, but they grew with greatly enhanced vigor on media which contained sucrose. The relative vigor of strain A.T.C. (original and one of its sucrose variants) is shown in figure 4. The plate was inoculated with a mixture of the two strains. It may be seen that there is not only a striking difference in the vigor of growth, but that secondary colonies which are developed in abundance on the original type are wholly lacking in the variant.

The permanence of the variants has not been determined fully, since the oldest strains have not been cultivated on sucrose-free media for a longer period than approximately three months. During this time ten transfers have been made, and at the same time tests for reversion have been made on sucrose media. Reversion has not occurred during this period in any of the several subraces tested. There has been no loss in capacity to attack sucrose, and secondary colonies are not produced. The variant races, when cultivated on plain agar, are not more vigorous than the original and do not appear to differ from it in any character except ability to ferment sucrose and lack of capacity to form secondary colonies.

The experiments to be reported next were directed at the cause of secondary colony formation in culture media which do not contain carbon compounds in the form of sugars or alcohols. Many such cases have been reported, but it is here that we approach the phase of our problem where little or no experimental evidence is available.

The cases reported by Preisz (1904) have already been mentioned. His explanation that the phenomenon is due to the germination of spores or other more resistant cells within the primary colony appears to have met with wide acceptance and has been frequently cited as the explanation for the resumed or secondary phase of growth.

Eisenberg (1906) described a type of secondary colony formation which he regarded as different in principle from that previously reported by Preisz. He believed that certain cells might become detached from the parent colony and make their way into the depths of the substratum, either by their own power, as in the case of motile forms, or by diffusion in other cases. Such cells may develop secondary foci of growth which first become visible as granulations and in certain cases develop into deeplying secondary colonies. These could not be seen in some species until after the surface growth had been scraped away. Such colonies always developed promptly (within the first or second day) and never at the surface of the primary growth. Whether this phenomenon is of common occurrence or not is impossible to say. Eisenberg believed that the nature of the culture medium was of some importance and that such colonies were more abundant when the medium contained either blood serum or egg albumin. Although he was careful to point out that the phenomenon differed from that described by Preisz, some recent writers have made no such distinction. Thus, Hadley includes the entire list given by Eisenberg in his compilation of secondary, colony-forming species. It is obvious that if such colonies originate in the manner described by Eisenberg they have nothing in common with secondary colonies which form only after the primary growth has reached the period of senesence, and that they are of no particular significance.

In order to determine the extent of secondary colony formation in the absence of specific sugars and alcohols, the several strains of *B. mycoides* were cultivated on a variety of culture media. These included agars prepared from casein digest according to the directions given by Kristensen (1925), tryptophane broth (desiccated bacto product) 1.0 per cent, peptone alone 1.0 per cent, bacto beef extract alone 1.0 per cent, and aminoids of beef 1.0 per cent. The results are shown in table 1 and figure 2.

It may be seen from the table that no secondary growth occurred on either beef extract alone or aminoids of beef, while on the digested casein media and peptone alone some strains proved consistently positive and others negative. It is a point of interest that six strains (A6, 17, 23, 402, 415, 407) which had previously been found negative on sucrose proved to be positive on the peptone and digested casein media, and that all strains (A2, C, 22, 94) which had previously been found positive on plain agar but negative on sucrose were positive on peptone and casein digest. With three exceptions (417, 421, 422) all strains which formed secondary colonies on sucrose agar were found negative on these media. It should be noted that these are the strains in which the direction of spiral growth and sucrose fermentation failed to correlate. Their behavior on peptone and casein digest shows that they respond to nutrients in a different manner from all other strains, since they alone were positive on sucrose and peptone. As in the case of sucrose agar, secondary colonies did not appear in any case until a period of from six to eight days had elapsed.

Various brands of peptone were tested, including bacto, Witte, Jensen, Fairchild, Parke Davis and proteose. No difference whatever could be observed in the case of the bacto, Fairchild and Parke Davis products, which proved to be the best suited for this purpose. Proteose peptone supported more vigorous growth, but the secondary colonies appeared less promptly and never became as large or well developed. Primary growth on Witte's peptone was less vigorous than with other products, and the phenomena of secondary colony formation were somewhat different. The primary growth failed to spread in the characteristic

fashion and had little resemblance to a colony of *B. mycoides*. Threads grew out eventually for a distance of about two centimeters as a very thin almost imperceptible halo. This was followed by development of secondary colonies which remained small, discrete and very numerous on the thin portion, but larger at the extreme margin where they became confluent and thus formed a wall or border. A second fringe developed which in time also produced secondary colonies. This same phenomenon was observed on some, but not all, of the strains on both casein digest and tryptophane agar. Jensen's peptone supported a more vigorous growth of all strains, but no secondary growth occurred. It thus resembled the beef extract and aminoids of beef rather than the other brands of peptone.

Subcultures were prepared from all positive strains by plating methods. In all cases the thread-like typical form was obtained, but the subraces grew with enhanced vigor and failed to produce secondary colonies either on the same or on a different brand of peptone.

When sucrose was added to 1.0 per cent peptone the strains which had produced secondary colonies on beef extract peptone agar containing sucrose were all found to be positive, while all other strains positive on peptone alone or peptone plus beef extract became negative. No secondary colonies were formed on 1.0 per cent peptone plus 2.0 per cent glucose, maltose, or glycerol, nor by any but the four already mentioned (A2, 62, 22, 94) when beef extract was added. In this case less than 0.2 per cent was not sufficient to suppress their formation in the remaining peptone-positive strains (A6, 17, 23, 402, 405, 407). The addition of 0.2 per cent sodium citrate, tartrate or acetate to 1.0 per cent peptone agar did not cause suppression of secondary colony formation in any of the peptone-positive strains.

These experiments show that secondary colony formation may be caused in some but not all strains by nutritive substances other than sugars and alcohols. This fact does not appear to have been fully appreciated in the past. Both peptone and casein digest are complex substances which contain some fractional parts which are more readily available as nutrients than

others. Until experiments are conducted with synthetic media and purified derivatives it will not be possible to designate the precise substances concerned. The results indicate that the more highly digested products, such as aminoids of beef, are more completely utilized by all of the cells and that variation is therefore less likely to occur.

The results presented thus far show that differences exist in the nutritive requirements necessary for the formation of secondary colonies by the several strains of *B. mycoides*. Such colonies may be produced under the following conditions: (1) when sucrose and protein derivatives are both in the medium but not on a medium otherwise identical which lacks sucrose (7 strains); (2) when the agar contains protein derivatives alone but not when sucrose or certain other readily available sources of carbon are also present (10 strains); (3) when protein derivatives alone are present, and in a medium of the same composition but containing sucrose also (3 strains). The presence of a non-utilizable carbon compound has no effect. The distribution of the strains has been previously discussed and is shown in the accompanying table.

It has also been shown that subraces from the secondary colonies vary from the parent race in capacity to attack the specific compounds which must be present to cause their origin and development. This variation does not appear to extend to other characteristics such as the form of the colony or size, shape, and structure of the cells. When cells were examined from the secondary and primary colonies, some differences could be noted, but when subraces which varied with sucrose were cultivated on sucrose-free medium and examined, they could not be distinguished from the original, when cultivated in the same medium. It is believed, therefore, that these differences in the morphology of cells taken directly from secondary colonies are no greater than can frequently be observed when the same strain of an organism is cultivated on different media. Secondary growth occurs on a medium which is quite different from that which supports the primary growth. This is due in part to changes which have occurred in the medium itself such as ac-

cumulation of metabolites, depletion of the nitrogen-bearing compound and desiccation. But the enhanced ability of the organism to utilize sucrose is probably of more importance than any changes which have taken place in the medium. It is not to be expected that an organism would show precisely the same details of morphology when cultivated on sucrose agar and on agar not containing sucrose. This is very well illustrated by a decrease in spore formation and by more pronounced tendency to pleomorphism shown by strains of *B. mycoides* when cultivated on media containing utilizable carbohydrates or alcohols. These same tendencies are shown by the secondary colonies borne on sucrose agar cultures. This point does not appear to have been fully appreciated and has doubtless led to some confusion in the past.

Since variation has been shown to occur in all of the cases of secondary colony formation considered here, the experiments which follow have been directed at its nature and its causes. Although variation has been studied since the earliest days of bacteriology, there is probably no phase of the subject concerning which there are so many conflicting opinions. There is no intention at present to treat of this subject in a general way, but it is necessary to consider the factors involved in the special type of variation which leads to the formation of secondary colonies.

There is little or no difference of opinion that enhancement of fermentative power occurs only when the organism is cultivated in contact with the specific compound with which the variation occurs. It is likewise recognized that the variation depends also on inherent potentialities of the organism, since no amount of contact can cause variation in all cases.

Henderson Smith (1913) expressed some doubt as to the validity of the former statement. He regarded it as more probable that *B. typhi*, which varies in dulcitol tends to produce variants even in plain broth, but since dulcitol is absent and the characteristic is not well pronounced at the beginning, it is lost through lack of a selective agent. There seems to be no experimental evidence which supports this theory.

A somewhat similar explanation but differing from it in some

important details is the well-known theory of "pure lines" or biotypes. According to this view, a mass culture of bacteria may consist from the beginning of some cells which have the fermentative capacity well developed and others which have no such capacity. The sugar or other compound serves merely as a selective agent which picks out the pure line which has been present from the beginning. Results obtained by single cell isolations from such forms as *B. coli-mutabile* seem to exclude the possibility of pure line selection in many cases.

A still different explanation for this type of variation is based on the theory that all cells within a mass culture are alike at the beginning, wholly unable to attack the compound, or to do so but feebly. Any enhancement of this power must, therefore, be due to a specific variation by some or all of the cells. According to this view, the sugar serves as a specific inciting substance which stimulates a feeble or latent metabolic capacity to activity. Since the variation is at the same time beneficial, it is perpetuated in successive generations. This view appears to be in harmony with many observed facts.

The earlier workers, Neisser (1906), Massini (1907), Burri (1910), Reiner Müller (1909), arrived at different conclusions as to whether the full fermenting capacity is gained abruptly, as in the mutations of higher plants and animals, or whether it occurs by a slow gradual process of enhancement. More recently Stewart (1927) concluded that variation is caused either by mutation, which occurs but rarely, or by Mendelian segregation in such cases as the so-called mutabile strains of bacteria. Mellon (1925) explained it as due to chromatic rearrangements caused by isogamous conjugation.

Some light is thrown on these questions by the behavior of *B. mycoides* strains when cultivated in media which contain sucrose. Mere contact with the sucrose for given periods of time, which might apparently be prolonged to any degree, was not found to cause variation. This was determined by the method of daily serial transplants on media containing sucrose. Strain A.T.C. was carried through a series of twenty-five daily transplants on tubes of sucrose agar without acquiring any enhance-

ment of power to ferment sucrose or any lessening of the time period required for secondary colony formation.

Similar behavior was noted on the giant colonies which grew on sucrose agar in petri dishes. After a period of five to six days, the colonies began to appear as minute papillae on the oldest portion of the colony. As these papillae enlarged to form the secondary colonies new papillae appeared daily on the zone immediately beyond, until the whole surface became covered with the mature secondary colonies. There was never any formation of papillae until the primary threads had reached an age of five or six days. When the behavior was first noted and before much attention had been given to the matter, it was thought that this denoted origin of the colonies from spores, but in view of some experiments yet to be reported this is no longer believed. Stewart (1927) reported that a strain of *B. coli-mutabile* could not be caused to form secondary colonies sooner by preliminary cultivation in lactose broth for several hours before plating. The colonies were obliged to reach a certain stage of maturity without change of environment before the papillae began to develop. Papillation could be hastened or delayed depending on the number of colonies present. This was thought to be due to a shortening of the growth phase due to environmental conditions. Large colonies required a longer period for papillation and it could be delayed indefinitely by serial transplanting at short intervals. Mellon (1925) reported results the reverse of this. He found that crowded colonies frequently failed to form secondary colonies and explained this as due to the reaction itself or some metabolic product associated with it.

Results reported by Drudgeon and Pulvertaft (1918), who studied the delayed fermentation caused by several strains of *B. coli*, show that the reaction was not hastened when the bacilli were transplanted twice daily in lactose broth for a period of ten days.

Twort (1907) was able to "train" various species so that after a time they became able to ferment compounds which at the beginning they could not attack. His method consisted of subculturing at rather long intervals. He reasoned that a sufficient

period must elapse during which the protein constituents of the medium would become depleted and that the organisms with any such capacity would then attack the sugar. That this procedure was based on a sound physiological principle so far as depletion of the peptone is concerned has subsequently been shown by Berman and Rettger (1918) who studied, by precise methods, the influence of carbohydrates on nitrogen metabolism of various bacterial species. As would be expected, a non-utilizable carbohydrate, lactose, had no effect on the protein metabolism of *Proteus vulgaris*, since the protein was completely broken down in its presence.

It does not of necessity follow that Twort's expectation of a subsequent attack on the sugar would always apply. Numerous attempts by others to duplicate his results on different strains of *B. typhi* have failed. This was explained in part by Penfold (1911) who found that the original strain employed by Twort did not ferment lactose but showed greater powers of fermentation for certain other compounds than was to be expected from a typical strain of *B. typhi*.

There is some evidence (Bronfenbrenner and Davis, 1918, and Kennedy, Cummings and Morrow, 1932), that daily transplants may in some cases cause enhancement of fermentation, but this is probably due to differences in capacity of the strains at the beginning. There is no doubt that this method is wholly without effect in the strains of *B. mycoides* which are considered here.

This could be explained in the case of *B. mycoides* on the assumption that secondary colonies arise from spores and that variation is in some way caused in the process of sporulation. Such an explanation has been suggested by Stewart (1927), who regarded variation as due to an autogamic method of reproduction which, in the spore-forming species, takes place in sporulation. But this is based on an assumption which has every appearance of being false.

The fact that *B. coli-mutabile* could not be caused to form secondary colonies until the primary colony reached a certain stage of development was explained by Stewart (1927) as follows:

Now it is just before or shortly after that the papillae appear and papillae are daughter colonies which continue the growth of the parent without any change of surroundings. Are we not justified in suspecting that they originate from individuals which have conjugated and that the intrinsic factor which arrests the growth of the colony is the completion of a cycle by an outburst of conjugation . . . . We can, therefore, admit that the growth and arrest of growth of the bacterial colony is determined by an intrinsic rhythm.

The principal evidence presented in support of this hypothesis was that the causes generally accepted to account for arrest of growth are not sufficient and that the papillation of colonies of *B. paracoli* on media containing lactose or of *B. coli-mutabile* on sugar-free media are not explained by the principle of beneficial variation. This evidence does not stand critical analysis. The factors involved in arrest of growth are well known and universally accepted and the papillation in question was doubtless due to some peptone fraction in the medium.

A better explanation can be offered which is in harmony with all of the observed phenomena and with fundamental physiological principles. Arrest of growth is due not to intrinsic but to extrinsic factors chief of which are depletion of nutrients and the accumulation of harmful metabolites. It is probable that no general law is applicable to all cases but that different extrinsic factors may cause arrest of growth in different organisms. The behavior of the so-called mutabile strains indicates that sufficient nitrogen remains to support growth in the presence of an available source of carbon. It appears then that in a complex culture medium containing more than one source of carbon (either peptone fractions alone or peptone plus a carbohydrate or alcohol) the more readily available source of carbon is attacked first while the less available or non-utilizable remains. It appears to be probable that no variation occurs until a sub-critical stage is reached when some or all of the cells begin to utilize the carbon compound. This sequence of events accounts for the fact that secondary colonies are not formed until the primary colony reaches a definite stage of development and explains the failure to obtain enhancement of fermentation by rapid serial transplants in a medium containing peptone and a carbohydrate.

Before Twort's theory of the depletion of preferred nutrients can be accepted as the final explanation for the secondary phase of growth it must be determined whether or not such a phase occurs in synthetic culture media containing single sources of carbon and nitrogen. Other species of bacteria are better suited for this purpose than *B. mycoides*. The behavior of *B. coli-mutabile* in a synthetic culture medium containing ammonia and lactose, or *B. paratyphi* B in a medium containing ammonia and raffinose, as the sole sources of nitrogen and carbon should afford interesting results. Since it appears to be true that variation in a complex medium results from utilization of residual nitrogen and the second carbon compound, it is quite probable that variation may be found to occur also in a synthetic medium containing the same source of carbon and a non-carbon bearing source of nitrogen. It is to be expected, therefore, that secondary colonies may be found to develop on simple synthetic agar.

A final factor of importance must be noted. In order for the secondary phase of growth to occur, the conditions, aside from available food, must have remained suitable for growth. Such conditions are probably never as satisfactory as in fresh media and it is doubtless true that in many cases growth ceases before a condition of critical nutritive stress is reached. The most important factor involved here is probably the change in hydrogen ion concentration, but other products of metabolism are doubtless of some importance.

According to this view, secondary growth within the primary colony is due to a beneficial, variational response to hunger. It is obvious that the variant cells, in some cases, obtain additional advantages such as reversal of an unfavorable alkaline reaction due to subsequent formation of acid by the variant cells. The secondary colony could be expected to follow the same general method of development as a primary colony and in turn reach its own senescent phases. Whether a second secondary phase occurs in all cases must be regarded as doubtful. Such a condition, designated as tertiary colonies, was reported by Preisz (1904) for *B. anthracis*, by Enderlein (1925), and Hadley (1927) for other species. Such tertiary colonies have not been seen in

any of the strains of *B. mycoides*. The formation of tertiary colonies would depend on whether cells of the secondary colony are capable of still further enhancement through variation and whether conditions within the colony have remained suitable for growth.

An experiment of some interest in this connection was performed to determine the effect of reaction and other metabolites on growth of the primary colony and the formation of secondary colonies. The original strains were cultivated in standard nutrient gelatin for a period of sixty days. The medium was then restored to full nutrient value by the addition of peptone and beef extract, but the reaction was left unchanged. This liquid, containing the original growth and metabolites, received 2.0 per cent of agar and was then sterilized at 15 pounds pressure. Each medium was inoculated with its homologous culture and poured into Petri plates or poured and then inoculated on the surface. The resulting colonies bore little or no resemblance to typical colonies of any of the strains. Streak cultures on the surface invariably formed a dense restricted mat with no tendency to spread over the surface. After about two weeks, secondary colonies developed on some of the strains but not on others (fig. 7). The colonies on seeded plates remained small and compact and could not be easily distinguished from true secondary colonies (fig. 3). It is thus shown that the changes which occur in the medium, aside from depletion of nutrients, influence the form of the secondary colony. It is also shown that digested gelatin contains substances which, like other digested protein, suffice for secondary growth. On a medium prepared in a similar manner but converted into agar at the end of two weeks, the form of growth was influenced somewhat but was more nearly typical. Again secondary colonies appeared (fig. 1). The strain illustrated (strain B) did not form secondary colonies on peptone alone or on peptone plus beef extract.

There has been a tendency to regard the secondary colony as arising in some way through a response to these subsidiary unfavorable conditions, but to disregard the nutrient relationships, the importance of which had been clearly indicated by the work

of Neisser (1906), Massini (1907) and others of the earlier workers. It seems very doubtful whether secondary growth could ever be due to the development of cells better able to withstand unfavorable conditions or which possessed greater vitality, as assumed by Preisz for *Vib. chlolerae* and *B. diphtheriae*, or by cells which are able to overcome an inhibition, Arkright (1930), unless the unfavorable condition or the inhibition is conceived of as due to deficiency in the food supply. Quite similar to this is the notion that salt in sufficiently high concentration may have the same effect. This has not been tested experimentally for *B. mycoides*, but Enderlein (1916) reported that secondary colonies were formed by *Serratia marcescens* on agar containing an excess of sodium chloride. The experiment could not, however, be confirmed by Ramchandani (1929). The principle of so-called drug fastness or enhancement of capacity to resist harmful chemicals would appear to offer a means of testing this matter. In any case the variation should correspond with the substance and in all cases which have been carefully studied the variation has been to a specific nutrient substance.

It may now be pointed out more definitely how the principles just enumerated correlate with and enable us to explain the behavior of the three groups into which the strains of *B. mycoides* may be separated. These groups have previously been shown to correlate with the composition of the culture medium. The strains of group one obtain their carbon principally from the nitrogen bearing compounds during the early phases of growth but are capable of variation in response to sucrose. Accordingly, when the period of nutritive stress is reached, variation occurs, and this results in the development of secondary colonies. The strains of group two behave in a similar manner when cultivated on agar containing protein derivatives alone, but in this case variation appears to be related to some unused fraction of the digested protein. When sucrose is added, no secondary growth occurs. This is due to the well known protein-sparing effect of a utilizable carbohydrate. The rapid fermenting strains of this group are not capable of further enhancement in fermentative power, and colonies are not formed due to response to sucrose.

Since the course of nitrogen metabolism is hindered by formation of acid and other metabolites, growth is completely arrested before the critical stage is reached and the protein fraction which, in the absence of sucrose causes secondary growth, is not attacked. The slow fermenting strains of this group utilize the carbohydrate, but have no power to become enhanced in power through variation. The strains of group three which are capable of secondary growth with or without sucrose, vary to the sucrose in preference to the protein. The addition of a non-utilizable carbon compound is without effect in all of the groups, since nitrogen metabolism goes on unhindered and the organism lacks the capacity to vary in response to the added carbon compound. This was found to be true not only for carbohydrates and alcohols but for organic acids as well.

Whether the variation occurs abruptly or by slow gradual process of change does not appear to be of any special significance, although it has been a matter of interest. If variation is due to isogamous conjugation, as assumed by Mellon (1925), or to autogamy, as claimed by Stewart (1927), then we would expect it to occur suddenly, as in the case of true mutation or Mendelian segregation; but if this does not occur, which is altogether more probable, and the enhancement is due simply to an awakening of a latent function, then either might occur. Although slight differences have been observed in pure subraces isolated from secondary colonies of different age, the results were somewhat conflicting and no definite conclusions are possible.

In accordance with the work of Preisz on sporogenic and non-sporogenic races of *B. anthracis*, it has generally been believed that secondary colonies arise from spores in the sporogenic species, but the evidence has been meager. Since all of the strains of *B. mycoides* included in this study were found to be about equal in capacity to form spores, none of the differences already reported could be due to this cause.

The sucrose variant type was never obtained by plating methods from five day sucrose agar cultures when suspended in water and heated to 85°C. for a period of ten minutes, but was invariably present when the suspension was plated before heating.

Similarly, five day cultures on sucrose agar plates held at 65°C. for a period of two hours failed to form secondary colonies, although the spores were capable of growth when transplanted to fresh media. We are obliged to conclude from these experiments that the secondary growth of *B. mycoides* is due not to spores but to vegetative cells.

This is not wholly surprising, and in the light of all preceding experiments it would, indeed, seem to be more probable on theoretical grounds alone. Among the considerations which favor the origin from vegetative cells are the following: (1) there is no real reason to suppose that spores would be more likely to germinate in the media which support development of secondary colonies than in others which do not; (2) it is unlikely that variation is in any way connected with the process of sporulation or that it could occur after the spore is formed; (3) the method of secondary colony formation appears to be identical with the same process in non-spore forming species such as *B. coli-mutabile*, and others; (4) the conditions which cause sporulation are not perfectly known, but there is no reason to suppose that such conditions would be favorable to germination; (5) if variation occurs before sporulation, the added advantage should result in continued vegetative activity.

GENERAL DISCUSSION

Although the method of secondary colony formation which has been reported for *B. mycoides* has long been known, there has been a general impression that its application extends only to a few special cases of enteric organisms when cultivated in the presence of certain carbohydrates or alcohols. The experiments reported here have shown a much wider application of the principle than has been fully recognized in the past, and cast some doubt on various explanations that are based on principles which are not so well established.

The conclusion has been reached by various workers that some special biological significance must be assigned to this phenomenon and that this is to be found in a method of reproduction other than ordinary fission. Although such explanations are

based more especially on assumptions rather than experimental investigation, a certain type of experimental evidence has been presented in some cases. It is desirable to subject some of the various explanations to critical analysis in the light of all the known facts.

Mellon (1922) writes as follows:

> As regards the biological significance of these secondary colonies, I might state tentatively as the result of observations covering several years that they represent a special stage in the development of the culture and are to be regarded as the macroscopic counterpart of what we speak of microscopically as pleomorphism.

In a later communication (1925) he speaks of *B. coli-mutabile* as a transitional or developmental stage between "the wild non-lactose fermenting *B. coli*" and the normal fermenting strain from which the latter arose. Pleomorphism, according to Mellon's well known view (1925d), is due to isogamous conjugation. In harmony with this view he considered *B. coli-mutabile* as a form derived from *B. coli* by a process of sexual reorganization resulting in the loss of capacity to ferment lactose. "The loss of *B. coli's* ability to ferment lactose means that the developmental phase bearing this character (the secondary colonies) is absent or undeveloped."

It is not made clear whether the presence of lactose was regarded as exerting any influence on the sexual process or not, but the impression is gained that the sexual method of reproduction leads to variation which becomes manifest by "phases" which may or may not ferment the sugars. There is no ambiguity, however, in the statement that "bacteria are fungi whose life cycle is not completely known or at least not always completely manifested under the conditions of observation." This is directly in harmony with the notion of a complex life history and cyclogenic phases analogous to the fruiting or so-called perfect stages of certain fungi.

A more definite explanation of the relation of sexual reproduction to secondary colony formation is found in the work of Stewart (1926, 1927). Starting with the general hypothesis by which he

explained growth and arrest of growth as a process of "intrinsic rhythm" involving a vegetative and reproductive phase, he arrived at the conclusion that bacteria follow a Mendelian manner of inheritance. According to this view, *B. coli-mutabile* or any other species of similar behavior is to be regarded as the heterozygous form which gives rise to homozygous dominant and recessive strains through segregation of characters. The subrace which ferments the sugar or alcohol but does not again vary in response to it is regarded as the homozygous recessive, while the race which neither fements the compound nor gives rise to a fermenting race is the homozygous dominant.

It might be admitted that the three types of colon bacteria, *B. coli*, *B. paracoli* and *B. coli-mutabile*, or the three groups of *B. mycoides* strains, behave in a manner suggesting segregation, but that this is merely a superficial resemblance is easily shown. All workers have reached the conclusion that variation in these cases can occur only when the specific compound is present in the culture medium. It is impossible to reconcile this fact with Mendelian inheritance as we know it in higher plants and animals. This is not, however, the only objection or even the most damaging which may be raised. There is no evidence that the "homozygous dominant" form ever arises in cultures from these mutabile strains. This was explained as due to overgrowth by other forms in the mixed population arising in the culture by segregation, but the explanation does not appear to be well founded.

In order for Mendelian segregation to occur there must be a mechanism for segregation and recombination similar in principle to that already well known for the genes of higher organisms. This mechanism is given in a second hypothesis of segregation and autogamic conjugation. In this process it is necessary to make the assumption that under the stimulus of the sugar "the dominant allelomorphs are dissipated (as primitive polar bodies) and the bacterium varies." Both this and the preceding hypothesis are based on assumptions which are obviously false.

Other workers who have attempted an explanation of secondary colony formation based on a different method of reproduction include Hadley, Delves and Klimek (1932) and Enderlein (1925).

According to the former, secondary colonies may be due to asexual reproductive bodies, gonidia, which are produced in certain cells at a period of "reproductive maturity." The gonidia may, by continued vegetative division, produce a secondary colony which is made up of cells unlike the parent type both morphologically and physiologically. Such colonies yield strains having cells of filterable dimensions, feeble growth, and low fermentative powers. Aside from the uncertainties which exist as to the method of reproduction by means of gonidia, it is difficult to see how such cells, which can be cultivated only with great difficulty, could find suitable conditions for growth as a secondary colony. There is no reason to believe that such a method of secondary colony formation is common.

Enderlein (1925) has written at considerable length on the secondary colony as a stage in a complete cyclogenic life history, which includes both a haploid and diploid phase, comparable to that which occurs in higher plants and animals. The diploid vegetative cells produce gonidia which, by reduction to the haploid condition, become gametes ("spermits and oits"). These are incapable of further multiplication as such, but union of the gametes in fertilization restores capacity for growth which now proceeds with renewed vigor, producing a secondary colony. Nothing is said concerning variation or the relation to nutrients. Enderlein's theory is not convincing, since little or nothing is known concerning the principles on which it is based and it fails to account for all of the known facts.

It is impossible to conclude that any other explanation of the secondary phase of growth has so much in its favor or is backed by such a large amount of experimental evidence as Neisser's theory of variation and adaptation to nutrients. It does not appear necessary to resort to uncertain hypotheses in order to account for a phenomenon which has every appearance of being but the manifestation of well-known principles of physiology and variation.

The biological significance of the secondary phase of growth can be interpreted on this theory as beneficial variation to a specific nutrient compound.

SUMMARY

Twenty strains of *B. mycoides* have been studied with reference to the factors involved in the phase of growth which results in the formation of secondary colonies.

The secondary phase of growth is due to depletion of preferred nutrients and subsequent utilization of unused substances by certain cells. Ability to attack unused nutrients is acquired through variation due to the specific stimulus exerted by the substance concerned. The substances found suitable for promoting secondary growth were sucrose and protein fractions of unknown identity contained in digested meat, casein, or gelatin.

The strains may be divided into three groups on the basis of variation. Group one varies in response to sucrose, but not protein; group two, to protein, but not sucrose; group three, to both.

The subraces established from secondary colonies show enhanced capacity for utilization of the compound to which variation occurred and do not again produce secondary colonies in its presence. The variants are relatively stable.

Variation does not involve other characteristics, such as form of the colony, shape, size or structure of the cells. There is no evidence that the secondary colonies in this species are due to methods of reproduction other than fission, or that they represent a special phase of a complex pleomorphic life history through which the organism must pass in a cyclogenic method of development.

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PLATE 1

The colonies are shown natural size in figures 4 and 5. All other colonies are magnified 3.5 times.

FIG. 1. Portion of a giant colony of strain B on agar prepared from gelatin culture two weeks old. The colony was photographed on tenth day.

FIG. 2. Colony of strain 421 at end of two weeks on bacto peptone 1.0 per cent.

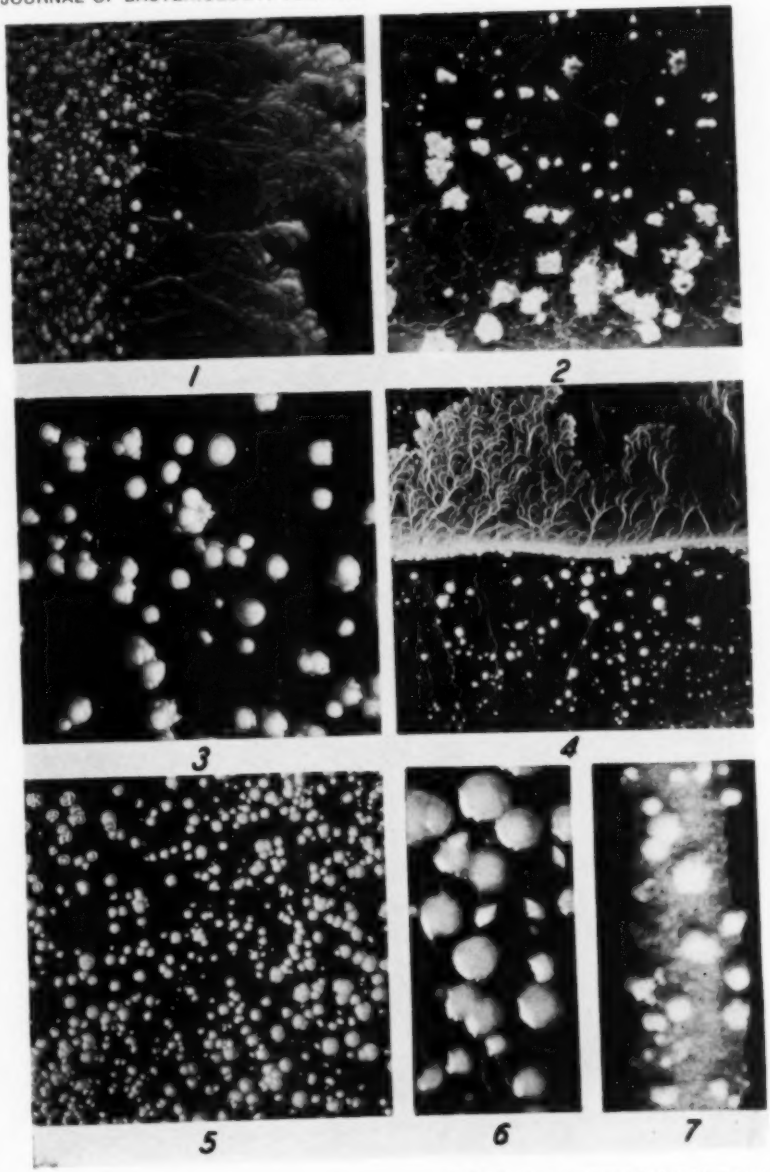
FIG. 3. Colonies of a poured dilution plate, strain A, on agar prepared from sixty-day gelatin culture by addition of agar 2.0 per cent, peptone 0.5 per cent, and beef extract 0.3 per cent. The plate was photographed at end of tenth day.

FIG. 4. Strain A.T.C. at end of two weeks on standard nutrient agar plus sucrose 2.0 per cent. Mixture of original and sucrose variant types.

FIG. 5. Strain A.T.C. original type on standard nutrient agar plus sucrose 2.0 per cent. Photographed at end of three weeks.

FIG. 6. Portion of the same colony shown in figure 5. The original threads show but faintly at this age.

FIG. 7. Strain A at end of thirty days on agar prepared as described for figure 3.



(I. M. Lewis: Secondary colonies of bacteria.)

THE BACTEROIDES OF HUMAN FECES

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Received for publication July 4, 1932

That human feces may contain a variety of species of non-spore-bearing anaerobic bacilli has been known for some time. Tissier showed in 1900 that the predominating organism in the stools of breast-fed infants was a Gram-positive bacillus without spores and strictly anaerobic, to which he gave the name of *Bacillus bifidus-communis*. In 1908, Tissier described four other anaerobes, also from infants' stools: *Coccobacillus preacutus*, *Coccobacillus oviformis*, *Bacillus ventriosus*, and *Bacillus capilosus*. Then, in 1911 and 1912, Distaso reported nine new species of non-spore-bearing anaerobic bacilli from adult stools: *Bacillus multiformis*, *Diplobacillus acuminatus*, *Bacilus variabilis*, *Bacillus pseudoramosus*, *Bacillus bullosus*, *Bacillus thetaiotaomicron*, *Bacillus variegatus*, *Bacillus cornutus*, and *Bacillus tenuis-spatuliformis* In 1912, Distaso's pupil Debono described *Bacillus tortuosus*.

Tissier, Distaso, and Debono isolated these organisms from stool cultures in deep glucose agar tubes, after the technic of Veillon and Zuber (1898). While they stated that certain species, such as *Bacillus bifidus* in infants, or *Bacillus variabilis* and *Bacillus thetaiotaomicron* in adults, were very common, they did not obtain any data concerning the actual numbers of these organisms per milligram of stool, nor did they ascertain what percentage of the viable, cultivatable organisms of the feces belonged to this anaerobic group. Because of this lack of quantitative data, the usual opinion among bacteriologists is that, in

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[2] A preliminary report of this work was made before the American Society of Experimental Pathology at the Philadelphia meeting, April 29, 1932. An abstract was published in the Archives of Pathology, Vol. 14, p. 279.

the adult at least, these species are rare and unimportant. Thus Ford (1927) states in his text book that "occasionally obligate anaerobes can be isolated by special technic, Veillon agar, etc." Other standard text books make no mention of these obligate anaerobes of adult stools, nor do the larger reference works, such as the Handbuch of Kolle, Kraus, and Uhlenhuth (1928), or the System of Bacteriology of the Medical Research Council (1929–1931). Very recently, Sanborn (1931) and also Torrey and Montu (1931) have observed that non-spore-bearing anaerobes or microaerophiles are frequently much more numerous in the stools of adults than are the aerobes; Sanborn states that the count obtained by planting high fecal dilutions in cooked meat tubes was frequently 300 times as high as that on aerobic plates.

Bergey's Manual of Determinative Bacteriology (1930) lists, under the genus Bacteroides, Tissier's *Bacillus bifidus* and his *Coccobacillus oviformis;* also the species described by Distaso and Debono. The genus Bacteroides is defined as consisting of "motile or non-motile rods, without endospores. Show good growth with ordinary culture media; without pigment formation. Obligate anaerobes." These species are also described in Ford's Textbook of Bacteriology (1927), and in Castellani and Chalmers' Manual of Tropical Medicine (1919).

In our own investigation, 65 stools were cultured; aerobic and anaerobic colony counts were made, and various anaerobic colonies from each stool were fished for replating and further study. Five of these stools were from infants; 40 were from normal adults; 20 were selected from patients that were apparently normal as far as their intestinal flora was concerned.

TECHNIC

Dilution of the feces. The method used by Torrey (1926) was followed. The fresh stool was emulsified in sterile 0.85 per cent salt solution. After allowing the coarse particles to settle, the emulsion was decanted into a sterile test tube having an inside diameter of 16 to 17 mm.; a heavy black pencil mark was made on the wall of the tube and the suspension diluted until the mark was just visible on looking through the tube toward a strong

light. This was dilution 1; from it, higher dilutions were made in four more tubes, each containing 9 cc. of sterile salt solution and 1 cc. of the previous dilution. Tube 4 therefore represents a 1:1000 dilution of tube 1. Torrey has calculated that a 4 mm. loopful from this fourth dilution represents approximately $\frac{1}{3600}$ mgm. of fresh feces of average water content.

Preparation and inoculation of plates. Our basic medium was beef infusion agar, containing 1.5 per cent of agar, 1 per cent of Parke Davis peptone, and 0.4 per cent of di-sodium phosphate. The pH was 7.6 to 7.8. Before pouring the plates, about 5 per cent of sterile blood and 0.15 per cent of sterile glucose (in the form of a 10 per cent solution) were added. With this small amount of glucose, the plates, being well buffered, do not become acid. This is important, as otherwise the more vigorously growing species soon make the medium unsuitable for those of slower growth. Our experiments indicate that this moderate alkalinity is most favorable for initiating growth of the Bacteroides. We tried several other media, including liver infusion, brain infusion, and coagulated egg, but our best results were obtained with the medium described above.

Plates were inoculated with a 4 mm. loopful of the fecal dilutions, the inoculum being well rubbed over the entire surface of the plate so as to obtain well distributed colonies. Anaerobic plates were made from fecal dilutions 3, 4, and 5; aerobic plates from dilutions 3 and 4.

Anaerobic cultivation. We have used two methods for securing anaerobic conditions for plates.

1. The double plate method, first described by Herrold (1920). We used *B. mesentericus* to consume the oxygen. Two covers (or two bottoms) of Petri dishes were chosen which had smooth perfect edges and were of exactly the same size. The glucose blood agar was poured into one and inoculated with the fecal dilution; a very stiff (3 per cent) nutrient agar was poured into the other and inoculated with *B. mesentericus*. Both plates were thoroughly dried in the incubator for 15 to 30 minutes; they were then placed face to face and sealed with two layers of adhesive tape. The double plate was incubated with the *B. mesentericus*

side up. To prevent leakage of air, the two plates must fit perfectly and be free from nicks. This method is widely used to produce a "partial oxygen tension;" it does more than that, however, as very excellent anaerobic conditions are produced (as well as a high concentration of CO_2, which is undoubtedly favorable in certain cases). It possesses the advantage of allowing inspection of individual plates. In our work, we incubated the cultures for 4 to 5 days. We used the double plates extensively in the early part of our investigation, but eventually abandoned them in favor of the second method. However, the most fastidious Bacteroides can be grown by this method; for instance, *Bacteroides bifidus*, which is considered difficult to isolate, can be grown directly from the stool.

2. The other method used by us was that of McIntosh and Fildes (1916), which depends upon the removal of oxygen by the union of hydrogen with oxygen under the influence of a catalyst, palladinized asbestos. For making the apparatus, we have followed the directions given by Zinsser (1930, pp. 151–153). A tube containing methylene-blue in alkaline glucose broth serves as an indicator for the absence of oxygen. This method is almost ideal, being clean, simple, inexpensive, and efficient. We have devised a slight modification which simplifies it still more by making the heating unnecessary. This is based upon the observation that the palladinized asbestos, when in the form of loose shreds, is a much more active catalyzer than when it is packed tightly on a spindle. We have placed about 0.3 gram of the loose palladinized asbestos in a small beaker and covered the top of the beaker with a fine meshed copper gauze, which is bent over and sealed around the edge with adhesive tape, so that there are no large leaks. The beaker is placed in the jar with the cultures and the hydrogen slowly run in. (We allow the hydrogen to bubble through a column of water, so that we can tell the rate of flow.) No heating is necessary; if the room is darkened, the projecting spicules of asbestos can be seen to glow when the hydrogen first enters. The usual precautions against introducing the hydrogen too rapidly must of course be observed. If the catalyser becomes sluggish, heating in the Bunsen burner flame restores its activity.

We now use this method for all of our anaerobic work. Cultures are incubated for 4 to 5 days without opening the jars.

Isolation of pure cultures. From 3 to 6 colonies from each stool were selected for pure culture study. Each strain was re-plated 3 times, single well isolated colonies being fished each time.

Stock cultures. Stock cultures of all of the Gram-negative and some of the Gram-positive Bacteroides were maintained on brain media. This was prepared by passing raw pigs' or sheep's brains through a meat chopper, and stirring up with enough broth to make a paste. The medium was then cooked in the Arnold sterilizer for 30 minutes, adjusted to pH 7.8, and 0.1 per cent of glucose added. It was then tubed and steamed in the Arnold sterilizer for 3 successive days. On this medium, most of the Bacteroides isolated grow out in twenty-four hours and remain viable in the refrigerator for 3 to 6 months. A few of the Gram-positive strains die off rather rapidly in this cooked brain; these are best maintained on blood agar slants.

Special media for subcultures. A 1 per cent peptone water containing 0.5 per cent sodium chloride and adjusted to pH 7.8 was used for fermentation tests. Brom cresol purple was used as an acid indicator. The sugars and other carbohydrates were made up in concentrated solution in distilled water, autoclaved, and then added to the broth tubes.

Milk was brought to pH 7.0 before sterilizing. This usually required 5 cc. of normal sodium hydroxide per liter of milk. If the milk is made more alkaline than this, it becomes yellow on sterilizing. If it is more acid, some of the Bacteroides fail to initiate growth.

Gelatin medium was prepared by adding 10 per cent of gelatin and 0.15 per cent of glucose to phosphate infusion broth and adjusting to pH 7.8.

Peptone water for the indol tests consisted of 1 per cent of Parke Davis peptone and 0.2 per cent of di-sodium phosphate, set at pH 7.8. When growth on this medium was poor, duplicate cultures were made on beef infusion broth.

Lead acetate broth was prepared by adding 1 cc. of sterile 1 per cent lead acetate solution to 200 cc. of sterile beef infusion broth, then distributing in tubes.

Coagulated serum slants were made by adding 1 part of 0.5 per cent glucose broth to 3 parts of serum and coagulating in the inspissator, then sterilizing in the Arnold sterilizer.

Coagulated egg albumen broth was prepared by cutting cubes of coagulated egg white, dropping into tubes of infusion broth, and sterilizing.

Cultures on the above media were not discarded as negative until they had incubated for 40 days or longer.

Indol tests. Twelve-day cultures were used for the indol tests. We used two tests on each culture: the Ehrlich test as given by Kligler (1914) and the Zoller (1920) test. The two tests confirmed each other in every case of our series.

AEROBIC AND ANAEROBIC COLONY COUNTS OF STOOL CULTURES

Both aerobic and anaerobic stool culture plates were incubated for 4 to 5 days; cultivation for a longer time did not increase the colony count. The anaerobic plates were given an additional day of aerobic cultivation, to allow any obligate aerobes to grow. The differences between the two sets of plates were usually very striking, as shown in table 1.

The anaerobic colony count, as given in table 1, includes the colonies of aerobes that developed on those plates; the true number of obligate anaerobes was computed by subtracting the aerobic colony count from the anaerobic colony count. The obligate anaerobes encountered in this series all have the characteristics of the genus Bacteroides. In these dilutions, spore-bearing anaerobes were never encountered.

Table 1 shows that in the majority of adult human stools, the predominating viable organisms are obligate anaerobes. This confirms the observations of Sanborn (1931) and of Torrey and Montu (1931). MacNeal, Latzer, and Kerr (1909) found that the anaerobic plate count of stools of the adult was, on the average, no higher than that of the aerobic plates; this we believe to be due to the fact that their period of incubation (48 hours) was too short, and their medium (litmus glucose agar) probably not favorable enough. Even under these conditions, they reported a few stools where from 2 to 12 times as many colonies developed on the anaerobic plates as on the aerobic controls.

From these 65 stools (60 of adults, 5 of infants) we isolated 198 strains of anaerobes for further study. Of these, 118 strains were Gram-negative and 80 were Gram-positive. We believe that the division of the Bacteroides into two main groups accord-

TABLE 1

Colony counts from plates inoculated with $\frac{1}{100}$ mgm. of feces from adults

NUMBER	AEROBIC COLONY COUNT	ANAEROBIC COLONY COUNT	NUMBER	AEROBIC COLONY COUNT	ANAEROBIC COLONY COUNT
1	15	56	31	5	38
2	6	48	32	150	167
3	0	65	33	2	48
4	7	80	34	1	42
5	5	76	35	4	141
6	17	55	36	5	85
7	2	80	37	26	43
8	12	150	38	24	72
9	30	104	39	12	152
10	58	145	40	60	154
11	27	320	41	4	54
12	14	116	42	67	88
13	7	75	43	0	16
14	8	62	44	6	40
15	62	195	45	4	50
16	12	95	46	61	124
17	10	145	47	12	64
18	2	87	48	20	60
19	1	42	49	5	53
20	53	162	50	2	42
21	2	38	51	10	160
22	4	89	52	24	124
23	8	116	53	10	167
24	12	72	54	2	70
25	12	36	55	1	74
26	3	42	56	12	42
27	4	41	57	120	126
28	4	74	58	8	158
29	86	138	59	16	132
30	39	59	60	15	93

Number of stools containing less than 50 per cent of anaerobes..... 5
Number of stools containing between 50 and 70 per cent of anaerobes..... 10
Number of stools containing between 70 and 80 per cent of anaerobes..... 6
Number of stools containing between 80 and 90 per cent of anaerobes..... 8
Number of stools containing 90 per cent or more of anaerobes..... 31

ing to the Gram stain reaction is a fundamental one, for the following reasons:

1. The Gram-positive Bacteroides in our collection either form bifid ends, or they grow in chains or pairs, or they have the aspect and arrangement of the diphtheria group. The Gram-negative Bacteroides are characteristically single organisms, often oval in shape, and frequently staining more heavily at the ends or around the periphery.

2. None of our Gram-positive Bacteroides ferment rhamnose. Many of the Gram-negative Bacteroides do ferment rhamnose.

3. A number of our Gram-positive Bacteroides ferment inositol, glycerol, or mannitol. None of our Gram-negative species ferment inositol; only one ferments glycerol and only one ferments mannitol.

4. Several of our Gram-positive Bacteroides reduce nitrates to nitrites; none of our Gram-negative species do this.

5. With two exceptions, all of our Gram-negative Bacteroides that ferment glucose likewise ferment galactose, levulose, and mannose; and with one exception, those that ferment lactose likewise ferment sucrose and raffinose. In the Gram-positive group, several species ferment glucose that fail to ferment galactose or mannose; and a number ferment lactose that fail to ferment sucrose or raffinose.

6. Most of our Gram-positive Bacteroides give a flocculent growth in infusion broth, with a clear supernatant fluid; most of them grow poorly or not at all in peptone water. The Gram-negative Bacteroides usually grow diffusely in infusion broth, and grow fairly well in peptone water.

For these reasons, we believe that the primary division of the Bacteroides into a Gram-positive and a Gram-negative group is a logical one.

The Gram-positive fecal Bacteroides seem to be made up of 20 or more species. Bergey's Manual (1930) lists 10. The descriptions in the original literature are often so meager that we have found it impossible to identify many of our strains with any of them. We feel it necessary to isolate and study many more

strains of the Gram-positive Bacteroides before we can classify them; hence we leave this group for a later report.

The 118 strains of Gram-negative Bacteroides fall into at least 18 species; 2 of these, *Bacteroides variabilis* and *Bacteroides thetaiotaomicron*, have been described by Distaso (1912); the remaining 16 species are new. We are certain that there are other species that we did not encounter, but our list probably includes all of the commoner fecal Bacteroides and some of the rarer ones.

Key to the species of Gram-negative fecal Bacteroides

A. No gas from peptone.
  B. Acid in arabinose and salicin.
    C. Acid in mannitol ..... *Bacteroides gulosus*
    CC. No acid in mannitol.
      D. Acid in rhamnose.
        E. Not capsulated ..... *Bacteroides thetaiotaomicron* (Distaso)
        EE. Capsulated ..... *Bacteroides variabilis* (Distaso)
      DD. No acid in rhamnose ..... *Bacteroides uniformis*
  BB. Acid in arabinose; no acid in salicin.
    C. Gelatin liquefied ..... *Bacteroides vulgatus*
    CC. Gelatin not liquefied ..... *Bacteroides incommunis*
  BBB. Acid in salicin; no acid in arabinose.
    C. Acid in xylose ..... *Bacteroides distasonis*
    CC. No acid in xylose ..... *Bacteroides uncatus*
  BBBB. No acid in salicin or arabinose.
    C. Acid in sorbitol ..... *Bacteroides tumidus*
    CC. No acid in sorbitol.
      D. Acid in rhamnose.
        E. Acid in lactose ..... *Bacteroides ovatus*
        EE. No acid in lactose ..... *Bacteroides vescus*
      DD. No acid in rhamnose.
        E. Acid in xylose ..... *Bacteroides convexus*
        EE. No acid in xylose ..... *Bacteroides exiguus*
AA. Gas from peptone.
  B. Acid in lactose.
    C. Acid in arabinose ..... *Bacteroides inaequalis*
    CC. No acid in arabinose ..... *Bacteroides insolitus*
  BB. No acid in lactose; acid in glucose ..... *Bacteroides varius*
  BBB. No acid in glucose.
    C. Milk coagulated ..... *Bacteroides coagulans*
    CC. Milk not coagulated ..... *Bacteroides siccus*

DESCRIPTION OF THE SPECIES OF GRAM-NEGATIVE FECAL BACTEROIDES

None of the Gram-negative Bacteroides studied by us attacked cellulose or fermented dulcitol, erythritol, or inositol. None of them reduced nitrates to nitrites. None of them were motile (except for Brownian movement). None of them attacked coagulated serum or coagulated egg albumen (in egg cube broth). None of them were pathogenic for white mice or rabbits when injected subcutaneously in large doses. These negative reactions, common to all, are omitted from the detailed descriptions of the species.

1. Bacteroides gulosus (isolated 7 times)

On blood agar plates, this organism appears as a small oval bacillus 1 to 2 micra long, 0.8 to 1 micron thick. It stains more deeply around the periphery. On glucose broth, the same oval forms occur, as well as various other forms: small cocci; bacilli with marked bi-polar staining; large swollen bacilli, 3 to 4 micra long, 2 to 3 micra thick, staining only at the ends; also longer bacilli up to 6 micra long.

Colonies on blood agar are soft, gray, entire, elevated, 2 mm. in diameter. On glucose agar the colonies are much smaller.

On infusion and glucose broth, the growth is heavy and diffuse. The pH reaches 4.8 on glucose broth.

Fermentation reactions: Acid and a very small amount of gas are produced from aesculin, amygdalin, arabinose, cellobiose, dextrin, glucose, galactose, glycogen, inulin, lactose, levulose, maltose, mannitol, mannose, melezitose, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose, and xylose. Sorbitol and mannitol requires 2 to 3 weeks for fermentation. Neither acid nor gas is formed from glycerol.

Gelatin is liquefied in 2 to 3 weeks. Milk is acidified and coagulated in 4 to 20 days. Indol is formed. Lead acetate is blackened.

This species differs from *Bacteroides thetaiotaomicron*, which it most closely resembles, in that it ferments mannitol and sorbitol and liquefies gelatin. It differs from *Bacteroides variabilis*

in that it is non-capsulated and in that it ferments mannitol, sorbitol, melezitose, and trehalose.

2. Bacteroides thetaiotaomicron (Distaso 1912) (isolated 14 times)

On blood agar plates, this organism usually appears as a small single oval bacillus, 1 to 2 micra long, 0.7 to 1 micron thick. It may stain solidly or only at the poles. In some strains, it appears as a slender rod, 2 to 4 micra long and 0.7 to 1 micron thick. In other strains, small coccoid forms predominate. In glucose broth, the bacilli are longer, up to 4 micra long, and often occur in pairs. Some large pale cells, about 3 micra long and 2 micra wide, staining only at the ends, may also be found in glucose broth cultures. No motility was observed; Distaso states that this organism is motile; we believe that he observed Brownian movement, which is very active.

On blood agar, colonies are soft, elevated, entire, and may vary from 1 to 4 mm. in diameter. They may slope straight up to a peak in the center, instead of being rounded. They may be grayish or yellowish. Glucose agar colonies are 0.5 to 1.0 mm. in diameter.

Growth on infusion broth and glucose broth is heavy and diffuse; on glucose broth, the pH reaches 5.0.

Fermentation reactions: Acid and (usually) a small amount of gas are formed from aesculin, amygdalin, arabinose, cellobiose, dextrin, galactose, glucose, glycogen, inulin, lactose, levulose, maltose, mannose, melezitose, raffinose, rhamnose, salicin, starch, sucrose, trehalose, and xylose. Four of our strains failed to produce gas from sugars. Neither acid nor gas is formed from glycerol, mannitol, or sorbitol.

Gelatin is not liquefied, even after 50 days of incubation. Lead acetate is blackened. Milk is acidified and coagulated in 4 to 12 days. Indol is formed. (Bergey's Manual states that indol is not formed; this is not in accord with Distaso's description.)

This species differs from *Bacteroides variabilis*, which it somewhat resembles, in not being capsulated, in not liquefying gelatin, in usually forming gas from fermentable sugars, and in fermenting melezitose and trehalose. It differs from *Bacteroides*

uniformis in morphology, in usually forming gas from fermentable sugars, and in fermenting rhamnose.

3. Bacteroides variabilis (Distaso, 1912), (isolated 8 times)

On blood agar, this organism may be quite round or oval, 1 to 2 micra in length. The periphery stains more heavily than the center. It has a heavy capsule. On glucose agar, one may see very long bacilli, up to 10 or 12 micra in length; these may be swollen, irregular in form, or curved. Stained with safranin or dilute gentian violet, the bacteria (especially when grown on glucose agar) appear reticulated. In glucose broth, oval and coccoid forms predominate; there are also swollen pale ovals, staining at one or both ends or in a band across the middle.

On blood agar, colonies are smooth, glistening, elevated, and very mucoid; they are about 1 mm. in diameter. Glucose agar colonies are pin point in size.

On infusion broth and glucose broth, growth is heavy and diffuse. The glucose broth reaches a pH of 5.0.

Fermentation reactions: Acid but no gas is produced from aesculin, amygdalin, arabinose, cellobiose, dextrin, glucose, galactose, glycogen, inulin, lactose, levulose, maltose, mannose, raffinose, rhamnose, salicin, starch, sucrose, and xylose. No acid or gas are produced from glycerol, mannitol, melezitose, sorbitol, or trehalose.

Gelatin is liquefied in 2 to 3 weeks. Lead acetate is blackened. Milk is acidified; some strains coagulate it in 25 to 35 days; others never coagulate the milk, though it coagulates immediately on boiling. (Distaso states that this species has no action on milk.) Indol is formed.

Bacteroides variabilis differs from the following species, *Bacteroides uniformis*, in that it is capsulated, in that it ferments rhamnose and does not ferment melezitose and trehalose, and in that it regularly liquefies gelatin.

4. Bacteroides uniformis (isolated 8 times)

On blood agar and on glucose agar plates and on glucose broth, this organism appears as a small single bacillus, 0.8 to 1.5 micron

long, with rounded ends. It stains most heavily at the poles and around the periphery; the bi-polar staining is best brought out with safranin. It is very uniform in size and appearance.

On blood agar, colonies are transparent, soft, elevated; they are 0.5 to 0.75 mm. in diameter. On glucose agar, they are pin point in size.

On infusion broth and glucose broth, the growth is diffuse. The glucose broth reaches pH 5.4.

Fermentation reactions: Acid but no gas is formed from aesculin, amygdalin, arabinose, cellobiose, dextrin, galactose, glucose, glycogen, inulin, lactose, levulose, maltose, mannose, melezitose, raffinose, salicin, starch, sucrose, trehalose, and xylose. Melezitose and trehalose usually require 12 to 14 days for fermentation. No acid or gas are formed from glycerol, mannitol, rhamnose, and sorbitol.

Two of our strains liquefied gelatin in 25 to 40 days; the other six failed to do so. Lead acetate is blackened very slowly or not at all. Milk is acidified and coagulated in 8 to 12 days. Indol is formed.

Bacteroides uniformis differs from the following species, *Bacteroides vulgatus*, in that it forms indol and in that it ferments amygdalin, cellobiose, melezitose, salicin, and trehalose, and in that it usually does not liquefy gelatin.

5. *Bacteroides vulgatus* (*isolated 38 times*)

This is the commonest species found in the feces of the adult.

On blood agar, this organism usually appears as an oval bacillus, 0.7 to 2.5 micra long. It usually stains solidly, though some stains will show bi-polar staining. One of our strains (M1) forms filaments up to 10 micra long. The bacilli are usually single, though some strains show a tendency to appear in pairs. This species is very variable in glucose broth: sometimes small oval bacilli are found exclusively; sometimes the bacilli are 4 to 8 micra long; or they may be swollen, vacuolated, or distorted or very irregular in shape. A few strains grow in chains of 3 to 6 elements in glucose broth.

On blood agar, the colonies are soft, translucent, grayish,

elevated; they are 1.5 to 2 mm. in diameter. About one-half of our strains are markedly hemolytic on blood agar plates; agar plates made up with washed red cells, however, do not show any hemolysis. With some strains, the colonies are tough and stringy, and hard to emulsify. Growth on glucose agar is as good as on blood agar.

In infusion broth and in glucose broth, growth is heavy and diffuse. The pH of the glucose broth reaches 5.0.

Fermentation reactions: Acid and a small amount of gas are produced on arabinose, dextrin, galactose, glucose, glycogen, inulin, lactose, levulose, maltose, mannose, raffinose, rhamnose, starch, sucrose, and xylose. Seven of our strains ferment aesculin, the others do not. Neither acid nor gas is produced from amygdalin, cellobiose, glycerol, mannitol, melezitose, salicin, sorbitol, or trehalose.

Gelatin is liquefied in 4 to 20 days (one strain, KA, which is typical in every other reaction, does not liquefy gelatin even after 50 days of incubation). Milk is acidified; some strains coagulate the milk in from 5 to 25 days; others fail to coagulate milk, though it coagulates promptly on boiling. Lead acetate is blackened. Indol is not formed.

Bacteroides vulgatus differs from the preceding species in that it does not form indol and does not ferment cellobiose, amygdalin, melezitose, salicin, or trehalose. It differs from *Bacteroides incommunis* in that it does not ferment amygdalin and cellobiose but does ferment glycogen and starch; and in that it liquefies gelatin.

6. Bacteroides incommunis

We have 2 strains, which differ slightly from each other.

On blood agar, the bacilli are single, 1 to 2.5 micra long, about 0.5 micra thick. The staining is solid. In glucose broth, they are swollen and irregularly vacuolated, and sizes vary from 1 to 3 micra long and 0.7 to 1.5 micra thick.

On blood and glucose agar, the colonies are about 1 mm. in diameter, elevated, and slightly yellowish. One strain forms soft colonies; the other is stringy when emulsified.

On infusion broth and on glucose broth, the growth is diffuse. The pH on glucose broth reaches 4.8.

Fermentation reactions: Acid and a small amount of gas are formed from amygdalin, arabinose, cellobiose, dextrin, galactose, glucose, inulin, lactose, levulose, maltose, mannose, raffinose, rhamnose, sucrose, and xylose. One of our two strains ferments glycogen and starch, the other does not. Neither strain ferments aesculin, glycerol, mannitol, melezitose, salicin, sorbitol, or trehalose.

Gelatin is not liquefied. Indol is not formed. Lead acetate is blackened. Milk is acidified but not coagulated; it coagulates promptly on boiling.

7. *Bacteroides distasonis* (*isolated 20 times*)

This species ranks next to *Bacteroides vulgatus* in frequency of occurrence.

On blood agar, this organism appears as a single, solid staining bacillus, 1.5 to 2.5 micra in length, 0.5 to 0.8 micra in thickness, with rounded ends. Some strains will show a few bacilli 5 to 8 micra long. The same forms appear on glucose agar and glucose broth.

The colonies on blood agar are soft, grayish, elevated, from 1 to 1.5 mm. in diameter. Two of our 20 strains are markedly hemolytic but not on agar made up with washed red cells. On glucose agar, the growth is poor, the few colonies that develop being pin point in size. Growth in infusion broth is light and diffuse. On glucose broth the growth is heavier and also diffuse. The pH reaches 5.2.

Fermentation reactions: Acid but no gas is formed from amygdalin, cellobiose, dextrin, galactose, glucose, inulin, lactose, levulose, maltose, mannose, melezitose, raffinose, rhamnose, salicin, sucrose, trehalose, and xylose. All but 5 of our 20 strains likewise ferment aesculin and all but 5 (though not the same 5) slowly ferment starch. No acid or gas are formed from arabinose, glycogen, glycerol, mannitol, or sorbitol.

Gelatin is not liquefied by 16 of our strains; the remaining 4 liquefy gelatin in 35 to 50 days. Indol is not formed. Lead

acetate is blackened. Milk is acidified, and all but 4 of our strains coagulate the milk.

Bacteroides distasonis differs from *Bacteroides vulgatus* in that it ferments amygdalin, cellobiose, melezitose, salicin, and trehalose but fails to ferment arabinose and glycogen; and in that it usually fails to liquefy gelatin. It differs from *Bacteroides communis* (which it resembles most closely) in that it ferments melezitose, salicin, and trehalose, while it fails to ferment arabinose.

8. Bacteroides uncatus (isolated once)

On blood agar, extreme variation in size and form occurs. Some of the organisms are very minute slender bacilli, 0.5 to 1.0 micron long; on the other hand, filaments 20 to 30 micra long are also found. The ordinary length is 5 to 8 micra. Curved and hooked forms are plentiful. Bacilli may be club shaped or fusiform or show other types of irregularity. On glucose broth, small slender bacilli, 1 to 2 micra long, with pointed ends, are the predominating forms.

Colonies on blood agar are very minute and transparent. Growth is very poor on glucose agar.

On infusion broth and glucose broth, growth is slow and light; the broth is clouded. Glucose broth reaches an acidity of pH 5.6 only after 8 to 20 days of incubation.

Fermentation reactions: After 8 to 30 days of incubation, acid but not gas is formed from dextrin, galactose, glucose, lactose, levulose, maltose, raffinose, rhamnose, salicin, starch, and sucrose. No acid is formed from aesculin, amygdalin, arabinose, cellobiose, glycerol, glycogen, inulin, mannitol, mannose, melezitose, sorbitol, trehalose, or xylose.

Gelatin is liquefied in 16 days. Indol is not formed. Lead acetate is not blackened. Milk is neither acidified nor coagulated.

This organism may be more common than our results indicate, as we have a number of times fished colonies that might well have been *Bacteroides uncatus*, but failed to obtain growth on transplant.

Bacteroides uncatus does not resemble any other species in our

series. We have classified it with *Bacteroides distasonis* because it ferments salicin; possibly *Bacteroides vescus* may be more closely related, as the latter also ferments rhamnose without fermenting xylose.

9. Bacteroides tumidus (isolated 4 times)

On blood agar, small thick oval bacilli predominate; they are solid-staining, occur singly, and are 1 to 1.5 micra long. On glucose broth, many swollen forms with irregular staining may be seen. These vary greatly in size; bacilli having the following dimensions were measured: 1 by 3 micra; 1.5 by 6 micra; 4 by 5 micra; 3 by 6 micra; 2 by 10 micra; 4 to 10 micra. The bodies of these swollen forms are usually very pale, with only the ends staining.

Colonies on blood agar and on glucose agar are soft, grayish, and elevated, about 1 mm. in diameter.

Growth is heavy and diffuse on infusion broth and on glucose broth. On the latter, the pH reaches 4.8.

Fermentation reactions: Acid but not gas is formed from dextrin, galactose, glucose, glycogen, inulin, lactose, levulose, maltose, mannose, raffinose, sorbitol, starch, and sucrose. Neither acid nor gas is formed from aesculin, amygdalin, arabinose, cellobiose, glycerol, mannitol, melezitose, rhamnose, salicin, trehalose, or xylose.

Gelatin is liquefied in 12 to 20 days. Indol is not formed. Lead acetate is blackened. Milk is acidified but not coagulated.

10. Bacteroides ovatus (isolated once)

On blood agar and on glucose agar and on glucose broth, this organism appears as a single small oval, 1 to 2 micra long, 0.5 to 1 micron thick. The staining is solid.

Colonies on blood agar and glucose agar are soft, grayish, elevated, 1 to 1.5 mm. in diameter.

Growth is diffuse and heavy on infusion broth and glucose broth. On the latter, the pH reaches 4.8.

Fermentation reactions: Acid and a small amount of gas are formed from aesculin, amygdalin, cellobiose, dextrin, galactose,

glucose, glycogen, inulin, lactose, levulose, maltose, mannose, raffinose, rhamnose, starch, sucrose, and xylose. Neither acid nor gas is formed from arabinose, glycerol, mannitol, melezitose, salicin, sorbitol, or trehalose.

Gelatin is liquefied in 4 days. Milk is acidified and coagulated in 4 days. Lead acetate is blackened. Indol is formed.

11. Bacteroides vescus (isolated once)

On blood agar, glucose agar, and glucose broth, this organism appears as a slender pointed bacillus, 1 to 2 micra long, sometimes slightly curved, with bi-polar staining.

Colonies on blood agar are very minute and transparent; usually there is no growth on glucose agar.

Infusion broth and glucose broth are diffusely clouded; the pH on glucose broth reaches 5.6.

Fermentation reactions: Acid but not gas is formed from cellobiose (in 30 days), dextrin, glucose, maltose, mannose, and rhamnose. Neither acid nor gas is formed from aesculin, amygdalin, arabinose, galactose, glycerol, glycogen, inulin, lactose, levulose, mannitol, melezitose, raffinose, salicin, sorbitol, starch, sucrose, trehalose, or xylose.

Gelatin is liquefied in 8 to 25 days. Milk is neither acidified nor coagulated. Lead acetate is not blackened. Indol is not formed.

12. Bacteroides convexus (isolated 5 times)

On blood agar and on glucose agar, this organism appears as a thick oval bacillus, 0.8 to 1.5 micra long. It occurs single or in pairs. On glucose broth it is usually 2 to 3 micra long.

Colonies on blood agar and glucose agar are elevated, grayish, somewhat opaque. They are 1 to 1.5 mm. in diameter.

Infusion broth and glucose broth show heavy diffuse growth; the pH of the latter reaches 4.8.

Fermentation reactions: Acid and a small amount of gas are formed from aesculin, amygdalin, cellobiose, dextrin, galactose, glucose, glycogen, inulin, lactose, levulose, maltose, mannose, raffinose, starch, sucrose, and xylose. No acid or gas are formed

from arabinose, glycerol, mannitol, melezitose, rhamnose, salicin, sorbitol, or trehalose.

Gelatin is liquefied in 20 to 30 days. Lead acetate is blackened. Milk is acidified and coagulated in 4 days. Indol is not formed.

13. Bacteroides exiguus (*isolated twice*)

On blood agar and glucose agar and on glucose broth, this organism appears as a very small slender bacillus, 0.5 to 1.0 micron long, occurring singly and in pairs.

Colonies on blood agar are of two types: the first type is pin point in size; the second type is large, gray, and moist, 1 to 1.5 mm. in diameter. On glucose agar, no growth occurs except where the inoculation is very heavy; here a thick moist film develops.

Infusion broth and glucose broth are diffusely clouded; the glucose broth reaches pH 5.6.

Fermentation reactions: Acid but no gas is formed from galactose, glucose, lactose, levulose, maltose, mannose, sucrose, and trehalose. One of our two strains likewise ferments raffinose. Neither acid nor gas is formed from aesculin, amygdalin, arabinose, cellobiose, dextrin, glycerol, glycogen, inulin, mannitol, melezitose, rhamnose, salicin, sorbitol, starch, or xylose.

Gelatin is liquefied in 16 to 20 days. Milk is acidified and may or may not be coagulated in 35 to 40 days. Indol is not formed. Lead acetate is not blackened.

14. Bacteroides inaequalis (*isolated once*)

Marked pleomorphism occurs on blood agar. Some forms are coccoid, 0.5 micron in diameter; others are slender filaments, often curved or hooked, 3 to 12 micra long. On glucose agar and on glucose broth, small ovals, 1 to 2 micra long, predominate. These show bi-polar staining and (in glucose broth) may form short chains.

Colonies on blood agar are pin point in size. There is no growth on glucose agar. Infusion broth and glucose broth are diffusely clouded; the glucose broth reaches a pH of 5.2.

Fermentation reactions: This species, as well as the succeeding ones, has the rather unusual characteristic of forming gas from peptone water, in the complete absence of carbohydrates. Only a small amount of gas is formed (about 5 per cent of the closed arm of a Smith fermentation tube). None of this gas is absorbed by alkali.

Acid, but no additional gas, is formed from aesculin, amygdalin, arabinose, galactose, glucose, lactose, levulose, maltose, mannose, raffinose, salicin, sucrose, and xylose. No acid is formed from cellobiose, dextrin, glycerol, glycogen, inulin, mannitol, melezitose, rhamnose, sorbitol, starch, or trehalose.

Gelatin is not liquefied in 45 days. Lead acetate is blackened. Milk is acidified but not coagulated. Indol is formed.

Like the succeeding 4 species that form gas from peptone, this species rapidly decolorizes the sulphonphthalein dyes brom cresol purple and phenol red, in the presence of meat infusion; slowly or not at all in peptone water.

15. Bacteroides insolitus (isolated once)

On blood agar and glucose agar and in glucose broth, the predominating form is a short thick bacillus, 1 to 2 micra long; there are often some slender, curved bacilli, 2 to 3 micra long.

Colonies on blood agar and glucose agar are minute and transparent.

Growth in infusion broth and glucose broth is heavy and diffuse; the pH in glucose broth reaches 5.4.

Fermentation reactions: A small amount of gas is formed from peptone. Acid but no additional gas is formed from galactose, glucose, glycerol, lactose, levulose, maltose, and mannose. Acid is not formed from aesculin, amygdalin, arabinose, cellobiose, dextrin, glycogen, inulin, mannitol, melezitose, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose, or xylose.

Gelatin is not liquefied in 45 days. Milk is acidified and coagulated in 30 to 35 days. Lead acetate is blackened. Indol is formed. Brom cresol purple and phenol red are rapidly decolorized in the presence of meat infusion.

16. Bacteroides varius (isolated twice)

On blood agar, slender bacilli, 1 to 2 micra long, predominate. On glucose agar, the bacilli are longer and thicker, being 2 to 3 micra long; the staining is very uneven, barred and vacuolated forms being common. In glucose broth, the bacilli are oval or coccoid.

The colonies on blood agar appear like very flat cones, 2 to 3 mm. in diameter. On glucose agar, the colonies are 1 to 2 mm. in diameter. Infusion broth and glucose broth are diffusely clouded. The pH of the latter reaches pH 5.0.

Fermentation reactions: Gas is formed from peptone. Acid and considerable additional gas is formed from galactose, glucose, levulose, and mannose. No acid and no additional gas are formed from aesculin, amygdalin, arabinose, cellobiose, dextrin, glycerol, glycogen, inulin, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose, or xylose.

Gelatin is not liquefied in 45 days. Lead acetate is blackened. Milk is neither acidified nor coagulated. Indol is formed. Brom cresol purple and phenol red are decolorized in the presence of meat infusion.

17. Bacteroides coagulans (isolated once)

On blood agar, this organism is a minute oval bacillus, 0.5 to 1 micron long, with marked bi-polar staining. On glucose agar and on glucose broth, it is longer and more slender, 1 to 2 micra long.

Colonies on blood agar are 0.5 mm. in diameter, soft and transparent. On glucose agar, the colonies are of the same size, but very few colonies develop. The growth on infusion broth and on glucose broth is diffuse.

Fermentation reactions: A small amount of gas is formed from peptone. No carbohydrates are fermented with acid or gas production.

Gelatin is liquefied in 8 to 12 days. Milk is coagulated in 8 days without acid production; the coagulum partly redissolves

after 3 to 4 weeks. Indol is formed. Phenol red and brom cresol purple are decolorized in the presence of meat infusion.

18. Bacteroides siccus (isolated twice)

On blood agar and on glucose agar, this organism appears as a short thick bacillus, about 1 micron long. In glucose broth, it is coccoid and often grows in chains of 4 to 6 elements.

TABLE 2

The chief biological reactions of the Gram-negative Bacteroides

	SPECIES	GAS FROM PEPTONE	GAS FROM GLUCOSE	GLYCEROL	MANNITOL	SORBITOL	ARABINOSE	SALICIN	TREHALOSE	AMYGDALIN	CELLOBIOSE	GLYCOGEN	RHAMNOSE	XYLOSE	LACTOSE	LEVULOSE	GLUCOSE	GELATIN	MILK	INDOL	LEAD ACETATE
1	*B. gulosus*	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	*B. thetaiotaomicron*	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
3	*B. variabilis*	−	−	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+	±	+	+
4	*B. uniformis*	−	−	−	−	−	+	+	+	+	+	+	−	+	+	+	+	∓	+	+	∓
5	*B. vulgatus*	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	±	−	+
6	*B. incommunis*	−	+	−	−	−	+	−	−	+	+	∓	+	+	+	+	+	−	−	−	+
7	*B. distasonis*	−	−	−	−	−	−	+	+	+	+	−	+	+	+	+	+	∓	±	−	+
8	*B. uncatus*	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	−	−	−
9	*B. tumidus*	−	−	−	−	+	−	−	−	−	−	+	−	−	+	+	+	+	−	−	+
10	*B. ovatus*	−	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
11	*B. vescus*	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	−	−	−
12	*B. convexus*	−	+	−	−	−	−	−	−	+	+	+	−	+	+	+	+	+	+	−	+
13	*B. exiguus*	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	+	+	±	−	−
14	*B. inaequalis*	+	−	−	−	−	+	+	−	+	−	−	−	+	+	+	+	−	−	+	+
15	*B. insolitus*	+	−	+	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	+	+
16	*B. varius*	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+
17	*B. coagulans*	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
18	*B. siccus*	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+

On blood agar and on glucose agar, the colonies are 1 to 1.5 mm. in diameter; they are elevated and dry and difficult to emulsify. In infusion and in glucose broth, growth occurs as a powdery sediment with a clear supernatant fluid.

Fermentation reactions: Gas in small amounts is formed from peptone. Acid, but no additional gas, is formed from levulose only.

Gelatin is not liquefied. Milk is not changed. Lead acetate is blackened. Indol is not formed. Phenol red and brom cresol purple are decolorized in the presence of meat infusion.

The chief biological characteristics of the above 18 species of Bacteroides are given in abbreviated form in table 2.

ATTEMPTED SEROLOGICAL CLASSIFICATION OF THE GRAM-NEGATIVE BACTEROIDES

We immunized 33 rabbits with different strains of Gram-negative Bacteroides, hoping that agglutination reactions would aid in the identification and classification of these organisms. We were unable to secure agglutinating sera of high potency, even after 15 to 18 semi-weekly injections; most of the sera agglutinated the homologous organisms in dilutions of 1:50 to 1:100.

To our disappointment, we found that these sera were strain specific rather than species specific; a serum rarely agglutinated any organism but the homologous one; when a serum did agglutinate another strain, absorption of the serum with the heterologous organism failed to remove the major agglutinin.

For example, we immunized 9 rabbits with 9 different strains of *Bacteroides vulgatus*, the commonest organism in our series. Each serum was tested against the 9 strains. In 5 cases, only the homologous strain was agglutinated. The other 4 sera each agglutinated one other strain besides the homologous one. These 4 sera were each absorbed twice by a heavy suspension of the heterologous agglutinable strain. Such absorbed sera still agglutinated the homologous strains though they now failed to agglutinate the heterologous ones.

In the same way, we prepared 4 sera against 4 strains of *Bacteroides distasonis*, and sera against 3 strains each of *Bacteroides thetaiotaomicron*, *Bacteroides variabilis*, and *Bacteroides uniformis*. In all cases the sera were strain specific.

DISCUSSION

Distaso (1912) has described two species of Gram-negative fecal Bacteroides that we did find in our stool cultures: *Bacteroides bullosus* and *Bacteroides variegatus*. His descriptions are given briefly as follows:

Bacteroides bullosus is a small rectangular bacillus with bi-polar staining. Young cultures show spherical or oblong forms that stain uniformly, also long slender rods with a thick enlargement at one end or the middle. Occasionally these forms bifurcate at one end. The organism is motile. Acid and abundant gas are produced from glucose. No action on lactose, sucrose, gelatin, or milk. Indol is not formed.

Bacteroides variegatus is sometimes a short bacillus, sometimes very long and flexuous. It is motile. It contains Gram-positive granules. Glucose and lactose are fermented without gas. Milk is coagulated. Indol is formed. Gelatin is not liquefied.

Bergey's Manual also classifies Distaso's *B. laevis* (1912), *B. liquefaciens* (1911), and *B. rigidus* (1911) in the genus Bacteroides. This must be an error, as Distaso clearly states that these three species are *facultative* anaerobes.

We did not meet any of the three species of Gram-negative non-spore-bearing anaerobes described by Tissier as occurring in the stools of infants. These three species are:

Coccobacillus anaerobius-perfoetens (1900). Description of this species is very meager; there are no data on indol production, liquefaction of gelatin, or action on lactose or sucrose. It is oval in shape, occurs single, in pairs or short chains, and produces acid and gas from glucose.

Coccobacillus preacutus (1908). This is a coccobacillus with long fine pointed extremities, the total length being 5 to 10 micra. It is rapidly motile. There is acid and abundant gas from glucose. No indol. No action on milk, gelatin, lactose, or sucrose.

Bacillus capilosus (1908). Appears as a large curved bacillus or tangled filaments. Non-motile. No indol. No action on gelatin, milk, lactose, or sucrose. Acid but no gas from glucose.

Harris (1901–1905) has described an interesting organism, *Bacillus mortiferus*, that obviously is related to our second group of Gram-negative Bacteroides because it produces gas from proteins without the addition of sugar. This was isolated from a liver abscess. It did not grow on ordinary media, the addition of blood, serum, or ascitic or hydrocele fluid being necessary.

SUMMARY AND CONCLUSIONS

1. In 91 per cent of our series of 60 stools of adults, the predominating organisms were obligate anaerobes of the genus Bacteroides. In the remaining 9 per cent, the aerobes were more numerous than the anaerobes. Over half of the stool cultures yielded 90 per cent or more of anaerobes.

2. The Gram-positive and the Gram-negative Bacteroides differ, biologically, in many respects. This justifies grouping and considering them separately. As our work with the Gram-positive group is incomplete, it will be reported at a later time.

3. The Gram-negative Bacteroides have been studied and classified. Two species, already described by Distaso, have been studied in greater detail. In addition, sixteen new species are described.

4. Agglutinating sera prepared against the Gram-negative Bacteroides are strain specific and are therefore of little or no value in the identification and classification of species.

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THE SIGNIFICANCE OF BRUCELLAR AGGLUTININS IN HUMAN SERA

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Received for publication, July 20, 1932

The attention recently directed to infection with brucellae (see Newman, 1928, 1929; Thompson, 1928; Bloxsome and Davey, 1929; Nightingale and Wilson, 1929; Dalrymple-Champneys, 1929, 1932; Hay, 1930; MacArthur and Wigmore, 1930; Scott, 1930; Williams and Sladden, 1930; Baxter, 1930, 1931; Wilson, 1932; Beattie, 1932) warrants further search for evidence of the infection in this country. In the serological diagnosis, however, difficulties may arise through repeated failure even late in the disease to demonstrate agglutinins in the sera of individuals proved to be infected by the isolation of the brucella (Burnet, 1924; Carpenter, Boak and Chapman, 1929), and through the agglutinative effects frequently shown by the sera of individuals who give neither clinical evidence nor history of undulant infection (Sedgwick and Larsen, 1913; Nicolle and Pratt, 1915; Evans, 1924; Hull and Black, 1927; Harrison and Wilson, 1928, and Bigger, 1931). These agglutinative effects are either specific or non-specific. If specific, the agglutinins may be actively produced by an attack of which the clinical features are absent, unnoticed or undiagnosed (see Shaw, 1906 and Vaccaro, 1908–9), or passively absorbed by the ingestion of milk containing agglutinins (Cooledge, 1916, and McAlpine and Rettger, 1925). Agglutinins actively acquired, however, probably disappear rapidly after clinical recovery (see Birt and Lamb, 1899; Evans, 1931; Hardy, Jordan, Borts and Hardy, 1931; and see below). Although the transference of antibodies by feeding has been frequently demonstrated (Ehrlich, 1892; Famulener, 1912; Little and Orcutt, 1922; Smith and Little, 1923); Evans, 1924, Carpenter, Boak and Chap-

man, 1929, and Bigger, 1931 believe that the passive absorption of brucellar agglutinins is unlikely. The possibility of the ingestion of living brucellae in the milk containing their agglutinins must of course be kept in mind. This ingestion would produce an active, in addition to a passive, immunity, and the latter would tend to mask the features of such an infection (see Schroeder and Cotton, 1911; Zwick and Krage, 1913; Kennedy, 1914; Evans, 1916, 1924; Huddleson, 1922; Wilson and Nutt, 1926; Morris, 1928; and Hasseltine and Knight, 1931).

The agglutinating effects of normal sera have rendered difficult the adoption of minimum titre as a criterion for diagnosis. The titres suggested include 10 (Nicolle, 1905), 30 (Bassett-Smith, 1906), 50 (Hull and Black, 1927), 80 (Cole, 1913), 100 (Kolle and Hetsch, 1911), 100 to 150 (d'Oelnitz and Liotard, 1924), and 200 (Fici, 1922). With the lower figures, non-specific effects may be erroneously regarded as specific and with the higher figures, a large proportion of true specific reactions may be missed (see Duncan and Whitby, 1930).

The method of investigating sera submitted for the Widal and Wassermann tests or taken from special groups of individuals has been widely pursued (see summary by Dible, 1932) and the percentage of positive results has depended on the titre accepted as indicative of infection or arbitrarily taken for experimental and statistical purposes. As would be expected, the proportion of sera positive at a dilution of 100 is greater in a population exposed to infection by diet or occupation than in a similar population not so exposed. The difficulty of determining a titre with diagnostic significance and the failure of a considerable proportion of known cases to show agglutinins at all, render desirable for diagnostic purposes a criterion other than the titre of the serum.

This paper records the agglutinating power for brucella emulsions of 2116 human sera obtained from the various dispensaries, clinics, hospitals and private patients in and around Liverpool. The great majority of the sera were submitted for the Wassermann test. In addition, by means of a questionnaire and various experiments with sera selected from over 5000 sera for their high titres, an attempt has been made to distinguish between specific

agglutination of brucellae and the agglutinative effects shown by the sera of many apparently healthy persons. The agglutinins of human beings known to be infected and of immunised rabbits were also investigated.

METHODS

In addition to the standard agglutinable suspensions of *Br. abortus* (Bang) and *Br. melitensis* prepared at the School of Pathology, Oxford, fresh suspensions in 0.85 per cent saline were prepared from forty-eight hours cultures of *Br. abortus* (Bang), no. 624, and *Br. melitensis*, no. 311, obtained from the National Collection of Type Cultures (Lister Institute). Variations in agglutinability were minimised by careful standardisation of the nasgar used to pH 7.6 (see Domingo, 1924). The suspensions were killed by exposure to 56°C. for thirty minutes and standardised to no. 1 of Brown's opacity standards (Brown, 1919).

It has been shown by Evans (1924) and Matthews (1924) that the titre of a serum for any organism varies inversely with the density of the suspension used. The suspensions were, therefore, not altered in any way during the experiments here described, in which comparison was made between the titre of a serum and the titres of the fractions and the titres of the serum after heating or after single or double absorption.

Fractioning of sera (see Liefmann, 1909; Ledingham and Dean, 1912; and Gibson, 1930). The carbonic-acid-insoluble fraction or "mid-piece" of the serum was precipitated from a mixture of 1 cc. of serum and 9 cc. distilled water by the action at 0°C. of carbon dioxide gas generated by hydrochloric acid and marble. In the earlier experiments the gas was allowed to pass through the mixture in a centrifuge tube, but later the mixture was placed in a small bottle and the air above it displaced with the carbon-dioxide. The bottle being closed and shaken, absorption of the gas took place. Several repetitions ensured complete saturation. This method obviated any loss of serum through excessive bubbling of the gas through the mixture and was found to give results identical with the method previously adopted. After the treatment with carbon dioxide the mixture was allowed to stand at 0°C. for one hour and was then centrifugalised. The clear supernatant fluid was pipetted off, made up to 10 cc. with distilled water, and 0.085 gram sodium chloride added, thus producing a 1:10 dilution in physiological saline of the carbonic-acid-soluble fraction or "end-piece" of the serum. An undiluted suspension of the deposit or insoluble fraction was produced by adding to it, drop by drop, 1 cc. of ice-cold saline. Washing of the de-

posit by alternately suspending it in distilled water and centrifugalising resulted in a loss, as shown by the end titre of its suspension, and was therefore discontinued.

After the action of the carbon dioxide, the precipitate continued to separate from the mixture for about twelve hours. Separation of the deposit at the end of one, six and twelve hours, however, showed by the titres when emulsified that nearly all the insoluble fraction had been precipitated by the end of the first hour.

Agglutination tests. Dreyer's tubes were used. They were immersed for twenty-four hours in a water bath at 37°C. A constant-level apparatus kept approximately one-third of the contents of each tube immersed (see Gardner, 1931). The stability of each emulsion in the absence of serum was controlled. The dilutions for whole sera and the carbonic-acid-soluble fractions ranged from 1:20 to 1:640 or higher, and for the insoluble fractions from 1:4 to 1:28 or higher.

Agglutinin absorption. The technique is not described, as the results were of no apparent value in differentiating between specific and non-specific effects.

EXPERIMENTS WITH SERA SUBMITTED FOR THE WASSERMANN TEST

The sera examined were dealt with in two groups.

Each of the first group of 2,116 sera was tested at a dilution of 1:30 with a suspension of *Br. abortus* 624. Two hundred and seventy-five sera showed complete or partial agglutination, i.e., 13 per cent. Admittedly, by the use of only one dilution for each serum, some sera capable of agglutinating *Br. abortus* in higher dilution may have been missed owing to the zone phenomenon, but the writer's desire was to obtain the maximum possible number of sera showing a high titre rather than to obtain accurate statistics as to the proportion of sera positive at a dilution of 1:30.

The following questionnaire was then submitted to each of the clinicians who had sent a positive serum to the laboratory.

Has the patient been abroad? If so, where?
Has the patient had Malta Fever? If so, when?
Has the patient had irregular pyrexia lately?
Is the patient's occupation in any way connected with cattle, milk or goats?

Has the patient recently had general malaise, night sweats or rigors?
Has the patient obvious anaemia or enlarged spleen?
If the patient is a female, has she ever aborted?
Has the patient received T.A.B. vaccine?
Does the patient drink unusual quantities of milk?

Of the 275 questionnaires sent out, 184 were returned completed. The remainder were not filled in owing to a variety of causes, such as the death of the patient, his discharge from hospital and return to sea. Of the 184, 91 had been outside the United Kingdom, but none had ever suffered, so far as is known, from Malta fever. The irregular pyrexia, general malaise, night sweats, rigors, anaemia or enlargement of the spleen of those who exhibited these features could usually be explained by some obvious pathological condition. Of the 184, 31 were, or had been, directly connected with cattle, 13 of the 31 having been employed on cattle boats; 4 of these had frequently drunk goat's milk and one buffalo's milk.

Relation to abortion. Sixty-six of the 184 were females, 4 of the 66 being under seventeen years of age. Of the remaining 62 women, 15 gave histories of one or more abortions or miscarriages. The sera of all but 3 of these 15 were negative to the Wassermann reaction but no complement fixation tests with polyvalent brucellar antigens were carried out with these sera (see Williams and Kolmer, 1917). Whitehouse (1929) considered that the abortion of the wife of a farmer whose stock was infected was definitely due to a brucella.

Relation to the Wassermann reaction. Two hundred twenty-one of the 275 sera which showed agglutinins for *Br. abortus* were negative to the Wassermann test; 53 gave a positive or doubtful reaction, and the remaining serum absorbed complement in the absence of antigen. The latter consisted of an alcoholic extract of sheep's heart to which cholesterol had been added (see Williams and Kolmer, 1917).

Relation to T.A.B. vaccination. The answers to the questions regarding the previous administration of T.A.B. vaccine were unsatisfactory, as many of the patients did not know whether

they had been vaccinated. Approximately a third of the 184 patients had probably received the vaccine.

Few of the patients admitted drinking unusual quantities of raw milk.

SERUM FRACTIONING

Towards the end of these experiments, considerable differences were found in the distribution of the agglutinins in the carbon-dioxide soluble and insoluble fractions. Gradually evidence accumulated that the ratio of the titres of the two moieties might be used for the diagnosis of recent infections in apparently uninfected human beings. Experiments were therefore carried out with known specific sera, i.e. from immunised rabbits and infected human beings. Later, a further series of "normal" human sera were studied.

EXPERIMENTS WITH RABBITS

Sera of 4 rabbits, nos. 2, 3, 5 and 6, were tested at dilutions of 1:2 up to 1:32 with the Standard Laboratory's suspensions of *Br. abortus* and *Br. melitensis*. The serum of no. 3 alone agglutinated the suspensions, the titres in each case being 1:2. Neither of the fractions of any of the four sera agglutinated either of the suspensions at dilutions of 1:2 or higher.

Rabbits 2, 3 and 5 then received intravenously, at intervals of four days, four or five increasing doses of suspensions of *Br. abortus* (624) killed by exposure at 56°C. for half an hour. Rabbit 6 was treated similarly with a killed suspension of *Br. melitensis* (311). All four rabbits were bled at the end of twenty-one, thirty-eight and seventy days. Table 1 gives the titres of the whole sera and their fractions for the two standard suspensions.

Immediately following immunisation, the agglutinins were located mainly in the soluble moiety (see table 1)—a result in keeping with those of Gibson (1930). By the seventieth day, however, the titres of the whole serum and of the soluble fraction in each case showed much larger reductions than the titre of the insoluble fraction. The ratios of the titres for *Br. abortus* of

the soluble and insoluble fractions immediately after immunisation were 20, 40, 40 and 80 respectively for rabbits 2, 3, 5 and 6, while forty-nine days later the corresponding ratios were $1\frac{1}{4}$, $2\frac{1}{2}$, $2\frac{1}{2}$ and 5.

TABLE 1

RABBIT	EMULSION	21 DAYS			38 DAYS			70 DAYS		
		Whole	Soluble	Insoluble	Whole	Soluble	Insoluble	Whole	Soluble	Insoluble
2	*Br. abortus*	1,280	2,560	128	320	1,280	64	320	80	64
	Br. melitensis	1,280	2,560	128	320	1,280	64	160	40	64
3	*Br. abortus*	2,560	2,560	64	2,560	800	128	320	160	64
	Br. melitensis	2,560	2,560	64	640	400	64	160	40	128
5	*Br. abortus*	10,240	20,480	512	640	320	32	160	160	64
	Br. melitensis	10,240	20,480	512	320	640	32	320	160	64
6	*Br. abortus*	640	10,240	128	320	640	32	*	20	4
	Br. melitensis	640	20,480	64	640	640	32	20	40	16

* No agglutination at 1:20.

EXPERIMENTS WITH THE SERA OF PATIENTS SUFFERING FROM ABORTUS FEVER

Through the courtesy of Drs. R. J. Minnitt and W. Tudor Evans, sera were obtained from two patients diagnosed on clinical grounds as suffering from abortus fever. The titres of the whole sera and their fractions for the standard laboratory suspensions are shown in table 2. In addition, the whole sera and their fractions were exposed to a temperature of 56°C. for a half hour, and their titres re-estimated.

In case 1, sample A was obtained about two weeks and B about thirty-six weeks after the onset of the illness. In both A and B the agglutinins were located mainly in the soluble fraction. The ratios of the soluble and insoluble titres for *Br. abortus* and *Br. melitensis* respectively were: for A, 20 and 10; for B, 5 and $2\frac{1}{2}$. These differences were due to the fact that during the thirty-four weeks the titre of the soluble fraction had dropped from 1,280 to 320, while that of the insoluble fraction had remained constant.

In case 2, the serum was obtained *at least* fifty-four days after the commencement of the clinical manifestations, and showed a fairly even distribution of the agglutinins between the two fractions.

The effect of heating was usually to lower slightly the titres of the whole sera of either of the fractions (see table 2)—a finding similar to that of Baxter (1932). Evans (1924), however, found a slightly higher percentage of positive sera in a series which had been heated to 56°C. for a half hour than in an unheated series. Dalrymple-Champneys (1929) quotes several workers who believe that heating eliminates non-specific agglutination.

TABLE 2

CASE NUMBER	EMULSION	WHOLE SERUM		CO_2 SOLUBLE		CO_2 INSOLUBLE	
		Unheated	Heated	Unheated	Heated	Unheated	Heated
1A	*Br. abortus*	1,280	640	1,280	640	64	32
	Br. melitensis	640	1,280	1,280	640	128	64
1B	*Br. abortus*	320	320	320	160	64	32
	Br. melitensis	640	320	320	160	128	64
2	*Br. abortus*	640	320	160	80	128	64
	Br. melitensis	640	320	160	80	128	64

The zone phenomenon was frequently observed in the experiments with both of the fractions as well as with the whole sera (Keefer, 1924; Spencer, 1930).

From the above experiments with the sera of immunised rabbits and infected human beings; it would appear that (1) early in an attack the specific agglutinins are mainly in the soluble fraction so that the "ratio" is high, and (2) that the soluble agglutinins decrease more rapidly than those in the insoluble moiety, thereby producing a rapid decrease in the "ratio."

EXPERIMENTS WITH WASSERMANN SERA SELECTED FOR THEIR HIGH TITRES WITH BRUCELLAR SUSPENSIONS

In the second group of sera, 3,500 samples were tested for agglutination at a dilution of 1:30 with a suspension of *Br. abortus*

(624). Forty positive sera were selected and the titres of each and of the fractions of each were determined for the Standards Laboratory's suspensions of *Br. abortus* and *Br. melitensis*. The ratio of the titres of the soluble and insoluble fractions was then calculated for each suspension, except when there was no agglutination in the lowest dilution (i.e., 1:20 for the soluble and 1:4 for the insoluble fraction). A questionnaire was sent to each of the clinicians concerned. In the series of 40 consecutive samples the titres for *Br. abortus* ranged from 20 to 160, and for *Br. melitensis* from 20 to 320. Owing to the large proportion of seafaring individuals among them, the results cannot be regarded as representative of the population of Liverpool.

Sera with titres of 160 or over

Seven of the sera (nos. 10, 11, 12, 17, 19, 29 and 38) showed a titre of 160 for at least one of the standard suspensions.

No. 10. A youth of seventeen years whose questionnaire was negative except for slight irregular pyrexia explicable by tuberculous cervical adenitis. The titres of his serum for *Br. abortus* and *Br. melitensis* respectively were: whole serum 160 and 40; soluble fraction 80 and 40; insoluble fraction 32 and 32; giving ratios of $2\frac{1}{2}$ and $1\frac{1}{4}$.

No. 11. Male, aged forty-eight. Died from tuberculous bronchopneumonia with abscess formation. Titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 160 and 160; soluble fraction 80 and 40; insoluble fraction 32 and 32; giving ratios of $2\frac{1}{2}$ and $1\frac{1}{4}$.

No. 12. Female, aged twenty-nine, suffering from gonorrhea. The answers to the questionnaire were negative except for the fact that she had worked in a dairy a year ago. Titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 160 and 320; soluble fraction 160 and 80; insoluble fraction 128 and 64 giving ratios of $1\frac{1}{4}$ and $1\frac{1}{4}$.

No. 17. Male, aged twenty-nine, suffering from syphilis. The answers to the questionnaire were negative except that he had been abroad and had received 3 doses of T.A.B. vaccine. Titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum, 80 and 160; soluble fraction 160 and 160; insoluble fraction 32 and 32, giving ratios of 5 and 5.

No. 19. Female, aged forty-three, suffering from tabes. She had had enteric fever sixteen years ago and in her youth had worked in a dairy.

Otherwise her answers to the questionnaire were negative. Titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 160 and 40; soluble fraction 80 and 40; insoluble fraction 128 and 64, giving ratios of $\frac{1}{8}$ and $\frac{1}{8}$.

No. 29. Female, aged forty-two, attending an ante-natal clinic. Her answers to the questionnaire elicited the facts that she frequently drinks a pint of milk, and out of four pregnancies three fetuses were born dead before term. The clinician has since reported that the latest pregnancy has terminated with an abortion. Serum negative to the Wassermann test. Titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 160 and 160; soluble fraction nil at 20 and nil at 20; insoluble fraction 16 and 8, giving ratios of at least less than $1\frac{1}{4}$ and $2\frac{1}{2}$.

No. 38. Male, aged forty-three, suffering from gonorrhea. Had been in Egypt and stated that he had had Malta fever in 1920 (twelve years ago). Had had T.A.B. vaccine. Otherwise his answers to the questionnaire were negative. Serum negative to the Wassermann test. Titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 80 and 160; soluble fraction 80 and 40; insoluble fraction 32 and 32, giving ratios of $2\frac{1}{2}$ and $1\frac{1}{4}$.

Sera with ratios of 10 or over

In only 4 of the 40 sera (nos. 1, 5, 9 and 36) were the ratios of the titres of the soluble and insoluble fractions as high as 10. No definite diagnosis had been made in any of the 4 cases. Incubation of the clot, from which the serum was separated, was made in each case, both aerobically and in an atmosphere of 10 per cent carbon dioxide (Wilson, 1930), but no brucellae were obtained. Little importance, however, need be attached to these negative results as the attempts at cultivation were only made four, or five, days subsequent to withdrawal of the blood from the patient.

No. 1. Female, aged thirty-three. Although she had never been abroad, her husband was stated to have had malaria in Cuba and Havana. Had aborted on three occasions. Serum negative to Wassermann Test. Intermittent pyrexia; profuse sweating at night; spleen unfortunately not examined. The titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 40 and 20; soluble fraction 80 and 40; insoluble fraction 8 and 4, giving ratios of 10 and 10.

No. 5. Seaman, aged twenty-four. Pyrexia of unknown origin (malaria excluded) in Manaos, South America, five years ago. Had

been frequently to South America and had just returned. Indefinite illness with fever, of which bronchitis was the main feature. Serum negative to Wassermann test. The titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 40 and 40; soluble fraction 40 and 40; insoluble fraction 4 and 4, giving ratios of 10 and 10.

No. 9. Female, aged nineteen. Marked anaemia. Serum positive (+) to Wassermann test (congenital syphilis). Negative answers to all the questions except that she had pyrexia and worked among goats. The titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 40 and 20; soluble fraction 160 and 20; insoluble fraction 16 and nil at 4, giving ratios of 10 and *at least* 5.

No. 36. Seaman, aged twenty-nine. Had been on Continent and South America. Pyrexia. After a completely negative examination, a diagnosis of neurasthenia had been made. Serum negative to the Wassermann test. The titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 40 and 40; soluble fraction 160 and 160; insoluble fraction 32 and 16; giving ratios of 5 and 10.

While there is no proof that any of these 4 cases were or had been infected with a brucella, the history and clinical examination of each is indefinite enough to be consistent with a diagnosis of such an infection. All had unexplained pyrexia and titres of only 40 for the whole sera do not exclude active brucellar infections. Indeed a considerable proportion of abortus cases fail entirely to show agglutinins (*vide supra*). In no. 1 the cause of the abortions was undiagnosed by the clinician; in no. 5, a definite diagnosis was not made; no. 9 was a congenital syphilitic with vague symptoms, and in no. 36 the diagnosis was "neurasthenia." These form a striking contrast to the remaining 36 cases which had ratios below 10 and in which, except for three (nos. 4, 34 and 40), a definite physical pathological condition was found. Numbers 4 and 40 were attending ante-natal clinics, and no. 34 had exposed himself to the risk of venereal infection.

No. 13 is interesting in that there had been very little risk of brucellar infection. The patient, para VII of fifty-one years, had varicose ulcers, had never been abroad, had no pyrexia, sweating, no abortions, no T.A.B. vaccine, and drank no fresh milk but used condensed milk in her tea. Serum negative to the Wasser-

mann test. Titres for *Br. abortus* and *Br. melitensis* respectively were: whole serum 80 and 80; soluble fraction 20 and 20; insoluble fraction 16 and 32, giving ratios of $1\frac{1}{4}$ and $\frac{5}{8}$.

Relation to gonorrhoea

It is remarkable that of the 40 patients, at least eleven (nos. 6, 12, 16, 20, 21, 24, 30, 31, 32, 38 and 39) had been infected with gonorrhoea, the actual number of those infected being probably considerably higher in view of the fact that five others were diagnosed as having syphilis (nos. 2, 7, 8, 17 and 25), 2 others tabes (nos. 19 and 23), and the Wassermann being positive in at least 3 others (nos. 4, 28 and 33) in addition to the congenital syphilitic (no. 9).

In view of this, the titres of the sera of individuals contracting gonorrhea were investigated. Through the courtesy of Dr. A. O. Ross, serum was obtained on three occasions from each of several cases. The first sample was taken immediately the diagnosis of acute gonorrhoea had been made; the second about ten days later, and the third on or about the twenty-first day, i.e., when the posterior urethra had had a chance of becoming infected. Owing to the frequency of cessation of attendance at the clinic after the first week or ten days, a large number of sera were examined before three samples were obtained from each of a series of patients.

It was found that when the first sample did not agglutinate the brucellae in dilutions of 1:4 and higher, no brucellar agglutinins could be demonstrated in the serum even three weeks after the appearance of the gonorrheal discharge. When, however, brucellar agglutinins were found in the first sample even in only low dilution, the titre might rise rapidly during the first three weeks of the disease. The ratio of the titres of the soluble and insoluble fractions, however, never rose to the high level typical of an acute attack of abortus fever. The titres for *Br. abortus* of a typical example are given below:

Serum taken when discharge appeared

Whole serum	8
Soluble fraction	8
Insoluble fraction	4
Ratio	2

Serum taken twenty-one days later

Whole serum	64
Soluble fraction	64
Insoluble fraction	32
Ratio	2

This heterologous response to the gonococcal antigen may be comparable to the high typhoid aggutinin content sometimes occurring in the serum of an individual who once had typhoid but whose blood no longer contained much agglutin until the individual was infected by some other organism or after a sharp febrile attack (Wells, 1929). Similar manifestations of apparently non-specific immunity reactions have been observed in therapeutic immunisation (Jobling, 1916 and Petersen, 1922), such as the reaction following the intravenous inoculation of typhoid patients with killed *B. coli*. Similarly, when an animal already immune to one protein is inoculated with a second, it develops precipitins for both these proteins and possibly for others with which it has not been immunised (Hektoen, 1917 and 1919). If a parallelism were legitimate, these results would suggest that the gonococcus might produce antibodies, either homologous or heterologous, to the antigens with which the individual had been previously infected. The increase of the brucellar agglutinins found during an attack of gonorrhoea does not therefore necessarily indicate that the individual has previously had a brucellar infection. The fact, however, that brucellar agglutinins have not been found to appear *de novo* during such an attack suggests a previous infection, or at least specific stimulation, in those cases in which the brucellar agglutinins are found. Havens and Mayfield (1931) believe that agglutinins develop as a result of specific antigenic exposure under natural conditions even without a clinical infection, and that "exposure to certain kinds of bacteria will start up again the reproduction of apparently unrelated antibodies such as have developed previously in response to an antigenic stimulus of a different nature." The interpretation of diagnostic agglutination tests must therefore be qualified by the possibility of previous antigenic exposure, a possibility not precluded by the absence of evidence or knowledge of previous exposure. Havens

and Mayfield believe that specific antibodies, being the result of exposure to an infection, are an index of the prevalence of that infection in the region studied.

There is no reason to believe that similar findings as to brucellar agglutinins may not be made for infective conditions other than gonorrhoea. This may explain the probably erroneous suggestion that a tuberculous infection *per se* may induce brucellar agglutinins (see reference by Dible, 1932).

From a survey of the literature it would appear that most workers would regard a titre of 200 as indicative of infection. The lower titres, however, are more difficult to interpret and with them it is suggested that a better criterion for diagnosis of present or recent infection is to be found in the ratio of the soluble and insoluble fractions. It is further suggested that even when the titre of the whole serum is low, a ratio of 10 or over is indicative of a present or at least a recent infection. Conversely, a ratio well below 10 would indicate either no brucellar infection at all or at most a long past infection. The evidence of other workers having opportunities of obtaining sera from individuals infected or exposed to infection by diet, occupation or residence in endemic areas is desirable for assessing the value of this suggestion which, if substantiated, would be of considerable importance from the epidemiological standpoint

SUMMARY

1. A study has been made of the occurrence, distribution and specificity of brucella agglutinins in the sera of human beings and immunised rabbits.

2. In a series of 2116 human sera submitted for the Wassermann test, 275 or 13 per cent showed complete or partial agglutination at a dilution of 1:30. By means of a questionnaire it was shown that 15 of 62 women showing agglutinins in their sera gave histories of abortions; 53 of the 275 sera gave positive or doubtful reactions to the Wassermann test and there was no obvious relation to T.A.B. vaccination.

3. In rabbits *recently* immunized by the intravenous inoculation of killed brucellae and in infected human beings, the specific agglutinins are located mainly in the carbonic-acid soluble fraction of the serum. Those in the soluble moiety decrease, however, much more rapidly than those in the insoluble portion.

4. Exposure of the sera and their fractions at 56°C. for a half hour—whether from infected or "normal" humans or immunised rabbits—was usually associated with slight reductions in the titres.

5. The zone phenomenon was observed with soluble and insoluble fractions as well as with whole sera.

6. In a series of 40 sera selected for their high titres for brucellar suspensions from over 5,000 routine Wassermann sera, the ratio of the titres of the soluble and insoluble fractions was as high as 10 in four sera. All of these four were obtained from individuals who may have been experiencing *recent* infections. The remaining 36 samples gave ratios below 10 and were usually associated with a definite pathological condition other than a brucellar infection.

7. A high ratio of the titres of the soluble and insoluble fractions may be characteristic of an early active or very recent infection and therefore a better diagnostic criterion than the titre of the whole serum when that is below 200. If further evidence accumulates to substantiate this conclusion, a high ratio would be of great value to public health officials in identifying present or recent infections in which the titre of the whole serum remains persistently low.

8. During an attack of gonorrhoea, an increase may occur in the brucellar agglutinins already present in the serum, but brucellar agglutinins have not been found to arise *de vovo* during such an infection.

The writer has to thank Professor J. M. Beattie for helpful advice throughout the investigation, and also the numerous clinicians who completed questionnaires and sent sera, especially Doctors H. H. MacWilliam and A. O. Ross.

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DETECTION OF MICROBIAL LIPASE BY COPPER SOAP FORMATION

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Received for publication August 1, 1932

Copper soap formation as a test for the hydrolysis of fat (Carnot and Mauban, 1918) may be used for the detection of microbial lipase.

METHOD

To standard nutrient agar in the tube add 0.5 cc. per tube of melted butter of good quality and sterilize in the usual manner. The melted fat agar is cooled to 45°C., the fat well mixed in and the medium poured at once into a cold petri dish. On solidification the medium is inoculated by the streak method with the organism under test, and incubated at suitable temperature for forty-eight hours. The plate is then flooded with a saturated aqueous solution of $CuSO_4$ for ten minutes and rinsed gently with water.

When the fat has been attacked conspicuous bluish-green streaks of insoluble copper soap appear on the plate. The intensity of the color is a rough gauge of lipolytic activity.

In the case of palmitin the reactions involved may be represented thus:

$$\underset{\text{Palmitin}}{2C_3H_5(OOCC_{15}H_{31})_3} \xrightarrow[\text{(Lipase)}]{} + \underset{\text{Water}}{6H_2O} = \underset{\text{Glycerine}}{2C_3H_5(OH)_3} + \underset{\text{Palmitic acid}}{6C_{15}H_{31}COOH} \text{ and}$$

$$6C_{15}H_{31}COOH + 3CuSO_4 = 3H_2SO_4 + \underset{\text{Copper palmitate (blue-green)}}{3Cu(OOCC_{15}H_{31})_2}$$

Among organisms giving positive tests are: *Staphylococcus aureus, Staph. albus, Sarcina lutea, Pseudomonas fluorescens,*

Penicillium expansum and other molds. Among the negative organisms are *Escherichia coli*, *Bacillus subtilis*, *Bacillus mycoides*, and yeasts generally.

Cottonseed oil or soy bean oil may be used instead of butter.

The method is well adapted to the classroom as well as to the research laboratory.

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A PIPETTE ELECTRODE FOR THE DETERMINATION OF pH IN BIOLOGICAL FLUIDS[1]

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Received for publication, July 17, 1932

Workers in the field of biology frequently find themselves confronted with the problem of making pH determinations when only small amounts of material are available. This is particularly true in the field of bacteriology, because most of the culture work is done in test tubes seldom containing more than 10 cc. of solution. Determinations such as those for sugar, aldehyde and titratable acidity are often made on this amount of material, which means that micro methods must be used in order to complete all of the tests. Since many of the sugars used in this field are costly, it is impracticable to employ large amounts of solution; and also, the large number of tubes used calls for great economy of space.

It was on account of the small amount of solution available that the pipette electrode here described was developed. It is very useful in making pH determinations on the same culture tube from day to day. One cubic centimeter of the growing culture may be removed aseptically for testing without materially altering growth conditions. Very little time need be consumed in making the determinations.

THE PIPETTE ELECTRODE

The pipette electrode is made by blowing a small bulb in the center of a 3-inch piece of ordinary pyrex tubing 6 to 8 mm. in diameter and sealing into the bulb a piece of flattened platinum wire (see fig. 1). As suggested by Watson (1927), Halton and

[1] Food Research Division Contribution No. 152.

Fisher (1928), and Sorg (1930), gold-plated platinum wire should be used when solutions containing much suspended matter are to be tested. Platinum wire ranging between sizes 18 and 22

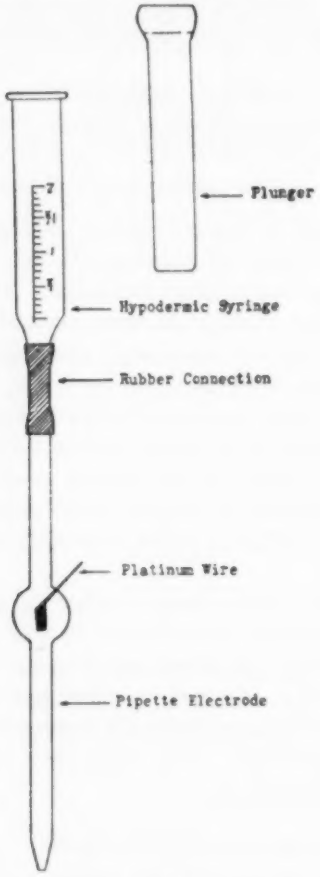

FIG. 1

should be used, in order to lessen the chance of its breaking; the wire should project into the center of the bulb and for about ½ inch outside. The seal should be fairly thick around the wire, to

avoid cracking of the glass upon cooling, and the pipette should be slightly drawn out at the lower end, to prevent dripping of the liquid employed in the test. The capacity of the completed electrode should be from 1.5 to 2 cc.

By means of a short piece of rubber tubing of convenient size the electrode is connected to a 5 cc. hypodermic syringe equipped with a spring holder which prevents the plunger from slipping down and forcing out the liquid. By means of this syringe the material to be tested can be drawn into the pipette electrode to any desired height. If a syringe is not available, a rubber bulb of suitable size may be used instead. By sliding the bulb down on the stem a point may be found at which the electrode just fills when the collapsed bulb is released.

PROCEDURE

The following procedure was employed when making pH determinations in growing bacterial cultures. One and one-half cubic centimeters of material were removed aseptically from a culture tube and placed in a small container made by cutting off the lower $1\frac{1}{4}$ inches of a test tube and mounting in a rubber stopper. Approximately 0.3 cc. of distilled water was added to increase the volume, since preliminary work had shown that this amount of dilution had no effect on the pH readings. Quinhydrone, either as dry crystals or as an alcohol-acetone solution, was thoroughly mixed with the sample and the mixture drawn up into the pipette electrode. The assembly was then suspended by means of a spring clip, with the tip of the pipette electrode dipping into a saturated KCl bridge. The lead wire from the potentiometer was fastened to the platinum wire by means of a Fanstock clip; potentiometer readings were then taken and the pH determined.

During the preparation of this manuscript the author found in the literature a description by Cullen and Biilman (1925) of a capillary electrode type D. While this electrode operates with a very small amount of solution, it would seem that dilution would take place rapidly, due to diffusion. The capillary bore of the electrode would prevent its use in any solution containing small particles of suspended material.

The pipette electrode here described does not have these disadvantages as the point of contact of the solution with the platinum wire is well removed from the area of dilution and the volume of solution in the bulb preserves the concentration of the test material around the electrode wire. The bore of the pipette is large enough to permit pH determinations on solutions which contain considerable suspended matter.

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SOME RESULTS ON THE USE OF CRYSTAL VIOLET IN BACTERIOLOGICAL CULTURE MEDIA FOR WATER ANALYSIS

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Received for publication, July 20, 1932

According to the present standard methods of the American Public Health Association, polluted water is indicated by the presence of Gram-negative non-sporeforming rods which ferment lactose with gas formation and grow aerobically on standard solid media.

Many workers have shown that positive results are often obtained with standard lactose broth from which members of the Escherichia-Aerobacter group are not isolated. This, it is believed, does not necessarily mean that members of this group are absent. Failure to isolate these organisms may often be due to the small number of colonies picked in routine work, or to the fact that these types may have been overgrown by other bacteria.

Gas production in lactose broth by spore-producing bacteria has been reported. Meyer (1918), Ewing (1919), and Norton and Weight (1924) isolated from water, aerobic spore-forming bacteria which produced gas from lactose. Kahn (1918) found in water anaerobic spore forming bacteria capable of producing gas from lactose broth. Sears and Putnam (1923), Holman and Meekison (1926), and others, have shown how false results in the analysis of water may be obtained through the symbiotic activities of the bacteria present.

Media to eliminate false results have been devised. The objects in view have been to inhibit the growth of bacteria which give false results, and to stimulate the growth of the Escherichia-Aerobacter group. Hall and Ellefson (1918) used gentian violet to eliminate the growth of aerobic and anaerobic spore-formers. Ruchhoft (1926), Hale (1926) and Jordan (1927) report favorable

results in eliminating false tests by using brilliant green and bile. Dominick and Lauter (1929) devised a phosphate-buffered medium containing brom cresol purple, methylene blue, and erythrosine to inhibit the growth of bacteria giving false results. Poe (1930) observed that crystal violet in a 1:75,000 dilution would eliminate most anaerobes. He found that the addition of bile destroyed the inhibitive power of the dye, so far as the growth of anaerobes was concerned. Salle (1930) proposed a phosphate-buffered medium containing crystal violet to eliminate the growth of organisms giving false results.

It was felt by us that the broth proposed by Salle (1930) contained the necessary constituents for supporting the growth of members of the Escherichia-Aerobacter group, and that the buffer content of this medium was a definite improvement over the ordinary lactose broth proposed by Standard Methods. The work of Leahy, Freeman and Katsampes (1931), using Dominick-Lauter medium, results obtained in this laboratory (not here reported), and well-known information on the acid limits of growth of members of the Escherichia-Aerobacter group, indicate the value of a phosphate-buffered broth for quicker production of gas and larger amounts of gas, in the analysis of water. It was believed, however, that the addition of any inhibiting substance to a medium to be used to support bacterial growth might also retard or inhibit the growth of the organisms which the medium was intended to detect. The important point in water analysis is to be certain to detect all polluted waters, regardless of being able to confirm all positive presumptive tests. The ability of Salle's broth to meet these requirements was tested by us.

Salle's medium for the presumptive test comprises a buffered lactose broth containing crystal violet in a concentration of 1:700,000. After being inoculated, the composition of Salle's crystal violet broth becomes:

Peptone	0.5 per cent
K_2HPO_4	0.5 per cent
KH_2PO_4	0.1 per cent
Lactose	0.5 per cent

Crystal violet in a concentration of 1:700,000.
Reaction unadjusted.

Salle, using pure cultures of *Escherichia coli* and *Aerobacter aerogenes*, reports approximately 100 per cent positive results with this medium. He fails to state the source of his cultures and the number of organisms per inoculum.

EXPERIMENTAL

In the work here reported pure cultures of Escherichia-type organisms were isolated from fresh feces of men and women in normal health. The stock cultures used, consisting of members of both the Escherichia and Aerobacter groups, were those carried in the culture collection of this laboratory. All fecal cultures used were shown by their Gram reaction, morphology, action in methyl-red medium and in lactose broth to belong to the Escherichia group.

The inoculum was prepared by so diluting forty-eight-hour broth cultures of the organisms that the number of bacteria per cubic centimeter would resemble the numbers that might be found in contaminated waters. The number of bacteria per inoculum was determined by plating on standard nutrient agar. Ten-cubic-centimeter inoculations were made directly from the dilution bottle to the medium contained in Smith fermentation tubes. Standard lactose broth was used as a control medium on all cultures tested. All inoculated tubes were incubated at 37°C. and observed at the end of twenty-four and forty-eight hours A Frost gasometer was used in measuring the amounts of gas formed.

A comparison of gas production in standard lactose broth and in Salle's crystal violet broth

Standard lactose broth and Salle's crystal violet broth were inoculated with laboratory stock cultures and recently isolated cultures of the Escherichia group. A summary of results obtained is presented in table 1. One hundred sixty-four Escherichia type cultures recently isolated from feces and seven stock cultures of the Escherichia-Aerobacter group were tested one or more times, making a total of 340 tests. The fecal cultures were obtained from 19 individuals.[1] Of these 164 fecal cultures,

[1] Cultures numbered from 1 to 30 were obtained from the same individual. Following number 30, each series of 10 was obtained from a different individual.

154 or 93.9 per cent failed to produce as much as 10 per cent gas in Salle's crystal violet broth in twenty-four hours, and 70 or

TABLE 1

Summary of results of gas production

CULTURE DESIGNATION NUMBERS	NUMBER OF CULTURES TESTED	NUMBER OF TIMES TESTED	STANDARD LACTOSE BROTH		SALLE'S CRYSTAL VIOLET BROTH				AVERAGE NUMBER OF BACTERIA PER INOCULUM
			Average gas in 24 hours	Average gas in 48 hours	Average gas in 24 hours	Number of 24-hour cultures negative	Average gas in 48 hours	Number of 48-hour cultures negative	
			per cent	*per cent*	*per cent*		*per cent*		
Stock	7	8	9	22	9	5	39	1	128
1–10	10	7	12	22	0	10	16*	10	82
11–20	10	3	14	24	0	10	4	9	82
21–25	5	1	7	15	0	5	4	4	71
31–40	10	1	5	18	0	10	0	10	29
43	1	2	23	36	0	1	0	1	185
51–55	5	2	10	17	0	5	45	0	64
61	1	2	12	17	0	1	45	0	38
71–80	10	1	20	28	0	10	16	4	26
81–90	10	1	17	24	3	8	46	0	52
91–100	10	1	16	26	1	10	52	0	55
101–110	10	1	21	29	9	5	24	4	86
111–120	10	1	15	22	7	7	41	0	66
121–130	9	1	13	24	0	9	43	0	40
131–140	8	1	3	11	0	8	27	0	22
141–150	10	2	12	18	0	10	35	2	49
151–160	7	1	5	16	0	7	23	2	36
161–170	10	2	5	18	0	10	7	10	29
171–180	10	2	5	19	0	10	38	0	29
181–183	3	2	9	28	0	3	42	0	62
191–195	5	1	4	14	0	5	5	4	21
201–210	10	1	8	19	0	10	0	10	50
Total or average...	164		11	21	1	154	24	70	56

* All cultures were negative one or more times, but were not all negative every time tested.

Totals and averages do not include stock cultures.

42.7 per cent failed to produce as much as 10 per cent gas in this same medium in forty-eight hours. In each instance, after forty-

eight hours' incubation, positive results were obtained in control tubes of standard lactose broth.

All tubes of crystal violet broth in which no gas was produced in forty-eight hours were incubated for a longer period at 37°C. Gas was finally produced in all tubes, some requiring as long as five days to produce 10 per cent gas. These results show that not all of the bacteria were killed, but that their growth was inhibited for a considerable length of time.

The number of bacteria in the inoculum, as determined by the agar plate method, shows that some of the tubes which produced no gas in crystal violet broth were inoculated with more bacteria than other tubes which produced a larger amount of gas. These results indicate that certain strains of the Escherichia genus are very sensitive to the inhibiting substance in Salle's crystal violet broth.

From these results, we are led to conclude that Salle's crystal violet broth has sufficient inhibitive action on Escherichia type cultures of fecal origin not only to prevent gas production by most cultures in twenty-four hours, but also to prevent gas production by many cultures in forty-eight hours.

The inhibiting action of crystal violet

Having determined that Salle's crystal violet broth exerted an inhibitive action on gas production by Escherichia-Aerobacter organisms, the inhibiting constituent in this medium and the degree of inhibition were investigated. Gas production in Salle's broth, prepared with and without crystal violet, was compared. Standard lactose broth was used as a control medium. Five stock strains of the Escherichia-Aerobacter group and 39 human fecal cultures of the Escherichia type were used. The inoculum was prepared in the manner previously described. The inoculated tubes were incubated at 37°C. and observed at the end of twenty four and forty-eight hours. The data obtained are presented in table 2.

In Salle's crystal violet broth four of the five stock strains and 29 of the 39 fecal cultures failed to produce any gas in twenty-four hours. A total of 37, or 84 per cent, of the cultures employed

failed to produce as much as 10 per cent gas in twenty-four hours. In this broth containing no crystal violet, all cultures produced an abundance of gas in twenty-four hours.

TABLE 2

The inhibitive action of crystal violet in Salle's broth

CULTURE DESIGNATION NUMBERS	NUMBER OF CULTURES TESTED	STANDARD LACTOSE BROTH		SALLE'S BROTH						AVERAGE NUMBER OF BACTERIA PER INOCULUM
				With crystal violet				Without crystal violet		
		Average gas in 24 hours	Average gas in 48 hours	Average gas in 24 hours	Number of 24-hour cultures negative	Average gas in 48 hours	Number of 48-hour cultures negative	Average gas in 24 hours	Average gas in 48 hours	
		per cent	*per cent*	*per cent*		*per cent*		*per cent*	*per cent*	
Stock	5	15	23	8	4	28	2	36	57	70
1–10	10	12	21	0	10	22	2	25	38	74
101–110	10	21	29	9	5	24	4	31	50	86
111–120	10	15	22	6	9	41	0	20	41	66
121–130	9	13	24	0	9	43	0	18	44	40
Total or average...	44	15	24	5	37	32	8	26	46	67

Totals and averages include stock cultures.

SUMMARY AND CONCLUSIONS

Salle's phosphate buffered broth, containing one part of crystal violet to 700,000 parts of water, has been tested. Seven laboratory stock strains of the Escherichia-Aerobacter group and 164 Escherichia type organisms recently isolated from human feces were used. Seventy of these fecal cultures, or 43 per cent, gave negative results after 48 hours' incubation at 37°C. All gave positive results in standard lactose broth.

The 164 fecal cultures used came from 21 samples, representing 19 persons. All tests made on the cultures from nine samples, representing nine persons, produced an abundant amount of gas in Salle's crystal violet broth in forty-eight hours. All tests made on the cultures obtained from three samples, representing three persons, failed to produce any gas in Salle's crystal violet broth in forty-eight hours. Of the remaining cultures, from nine

samples, representing seven persons, some gave positive results and others gave negative results.

The cultures producing negative results in forty-eight hours eventually produced gas, some requiring as long as five days for 10 per cent gas to be produced. The results show that the organisms were not killed, but that growth was delayed.

Gas production by members of the Escherichia-Aerobacter group was tested in Salle's broth with and without crystal violet. The inhibiting action was definitely shown to be exerted by the crystal violet.

In instances where crystal violet broth gave a positive test, a large amount of gas was produced, but usually little or no gas was produced during the first twenty-four hours.

These results have been obtained by testing seven laboratory stock cultures and 164 cultures isolated from 19 persons. They tend definitely toward the conclusion that Salle's crystal-violet broth should not be adopted as a standard test medium in water analysis. In order to maintain the proper margin of safety, no medium should be used which sometimes gives negative results in the presence of fecal pollution.

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BIOLOGY OF THE TUBERCLE BACILLUS

III. DOES THE SO-CALLED FATTY CAPSULE SERVE TO PROTECT THE TUBERCLE BACILLUS?

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Received for publication August 5, 1932

INTRODUCTION

It is well known that *Mycobacterium tuberculosis* is more highly resistant to physical and chemical agents than are most of the other non-spore-forming bacteria. However, it is less resistant than some of the Sarcinae which are also non-sporulating. While the cause of this variation in resistance is not well understood, it appears to be quite generally assumed that the superior resistance of the Mycobacterium group is related to the high percentage of fatty or waxy compounds contained in the bacilli. Indeed, the resistance is commonly ascribed to the so-called fatty or waxy capsule.

Just what is implied in the term "fatty capsule" is likewise uncertain. For present purposes we may regard it as the accumulated fatty material lying external to the cell sap. That these fatty and waxy compounds comprise a considerable fraction of the dry weight of the tubercle bacillus, commonly 25 to 40 per cent, is well known. It would be interesting and perhaps valuable to know whether these fatty bodies are related to the superior resistance of the tubercle bacillus. Several years ago, the Alice McDermott Foundation undertook experiments planned to solve this question if possible. The results are recorded in this paper.

A survey of the literature will not be attempted, since innumerable papers have been written which may be regarded as having a bearing upon this problem. Reference may be made to the excellent survey given by Wells, DeWitt and Long in their book

on The Chemistry of the Tubercle Bacillus (Williams & Wilkins Company, Baltimore, 1923).

Apparently it was Hammerschlag who determined, only six years after the discovery of the tubercle bacillus by Koch, that it contained an unusually high content of fatty compounds, reaching even to 40 per cent of its dry weight. This seems to be higher than in any other bacterium.

It is well known that the tubercle bacillus stains with great difficulty, but once stained it holds the dye with great persistance. Koch's discovery of the bacillus hinged upon the fact that he left his preparations in the staining solution for many hours thus permitting the organism to acquire sufficient dye to render it visible. It was a natural step to associate the resistance to stains with the high fat content of the bacillus, since fats are not stained by most dyes. Indeed, it was this characteristic that suggested the idea of a fatty capsule which prevented the stain from penetrating the organism. When it was found that the tubercle bacillus possesses a heightened resistance to chemicals generally, it was equally natural to ascribe this characteristic to the fatty capsule also; if it hindered the penetration of stains, it would likewise hinder the penetration of germicides. Buerger believed that the protective equipment of the bacillus rendered the germ less permeable to destructive agencies. Goris thought that the protective capsule was the predominating characteristic of the organism. Others have associated its pathogenic properties with this same characteristic.

METHODS EMPLOYED

Ehrlich and other early workers had made the interesting observation that very young tubercle bacilli are not acid-fast, a fact explained by the hypothesis that the young organisms had not had time in which to develop the fatty or waxy capsules. This work suggested the idea of growing fatless tubercle bacilli, an endeavor in which we have been engaged for several years. Up to the present time few workers have claimed success in growing fatless tubercle bacilli, and only one (Miller, Science, 1931) believes that he has found a stable fatless form. In our

work, we have never succeeded in growing truly fatless tubercle bacilli, but by resorting to non-pathogenic forms, we have been able to employ media devoid of glycerol, and thus grow organisms with a reduced lipin content. Table 1 presents some of the results obtained when these organisms were grown, with and without glycerol.

The results show that the bacilli may be grown with a greatly reduced fat content, the reduction ranging approximately between three-fourths and five-sixths of the normal content. These cultures having a reduced lipin content are for convenience designated as "lean bacilli," and they have been employed in the following investigation to determine whether they are less resistant

TABLE 1

Determinations of lipins in fat and lean tubercle bacilli

ORGANISM	CONDITION OF ORGANISM	WEIGHT DEHYDRATED	WEIGHT OF EXTRACT	WEIGHT OF LIPINS	LIPINS
					per cent
525	Fat	5.7615	1.4246	2.2235	31.17
525	Lean	0.3478		0.0319	9.20
525	Fat	3.2653	0.8207	1.3110	32.12
525	Lean	1.0093	0.1089	0.1020	8.75
520	Fat	2.4122	0.8734	0.9310	28.80
520	Lean	0.6470	0.1033	0.0573	7.76
520	Fat	4.6230	1.2165	1.3995	24.10
520	Lean	1.8409	0.1692	0.0981	4.94

to physical and chemical agents than are those having the higher lipin content. The cultures were used as young as possible, usually two to four day growths being employed.

The cultures were grown on agar containing meat extract and peptone for the lean, and on the same medium with the addition of 5 per cent glycerol for the fat bacilli. Ten successive transplants were made to stabilize the cultures as far as possible before they were used in the tests. Emulsions of the two cultures were made of approximately equal densities, and equal amounts of these emulsions were added to various concentrations of the chemicals for varying periods of time, or the emulsion was transferred to sterile bits of paper which, after drying, were exposed to

physical agents such as light and heat. Subcultures were made on glycerol agar to determine the viability of the organism. These subcultures were incubated for two weeks before recording a result as negative.

The culture selected was obtained from the University of British Columbia in 1925, and was designated as H520. It grows readily upon plain agar medium and is non-pathogenic. It appears doubtful, from other evidence, whether this culture has ever produced tuberculosis, but this can scarcely invalidate the results since the lipin content, and not the pathogenicity, is the important factor under consideration.

In carrying out the tests, another culture known as B525 was also used, thus duplicating all the data, but the latter are not reported since they are essentially the same. At times, cultures of *Staphylococcus aureus* and *Escherichia coli* were used as controls. While the latter gave results radically different from those for *Mycobacterium tuberculosis*, they did not serve to elucidate the problem and are therefore omitted.

RESULTS OBTAINED

Corrosive sublimate is so commonly employed as a disinfectant that it was decided to test its action on fat and on lean tubercle bacilli. The organisms were added to various concentrations of the chemical, and subcultures made after exposing them for varying lengths of time. The results are given in table 2.

The results show that both the fat and the lean organisms were killed by the corrosive sublimate in the same time in two trials, while in the third trial the lean bacilli were killed more quickly.

Phenol or carbolic acid was next tested for its action on fat and lean tubercle bacilli. The technic remained the same as for corrosive sublimate, except that the concentrations of the chemical were greater. The results are given in table 3.

In two trials the results indicate a slightly greater killing action for the lean bacilli. In all the trials the 1:400 concentration failed to kill; hence the results are omitted to simplify the table.

It was thought worth while to test the killing action of dyes upon fat and lean tubercle bacilli. Only two dyes were tried,

namely, gentian violet and acid fuchsin. Table 4 gives the results for the former.

The results show that exposures of one hour or less failed to kill in the concentrations tested. After twenty-four hours of

TABLE 2

Results when fat and lean tubercle bacilli were exposed to varying concentrations of corrosive sublimate

CONDITION OF ORGANISM	TIME EXPOSED	CONCENTRATION OF CORROSIVE SUBLIMATE		
		1:5000	1:100,000	1:2,500,000
	hours			
Fat	¼	+	+	+
	½	+	+	+
	1	0	+	+
	24	0	0	+
Lean	¼	+	+	+
	½	+	+	+
	1	0	+	+
	24	0	0	+
Fat	¼	+	+	+
	½	0	+	+
	1	0	+	+
	24	0	0	+
Lean	¼	+	+	+
	½	+	+	+
	1	0	+	+
	24	0	0	+
Fat	¼	+	+	+
	½	+	+	+
	1	0	+	+
	24	0	0	+
Lean	¼	+	+	+
	½	0	+	+
	1	0	0	+
	24	0	0	+

exposure, 1:5000 failed to kill the fat organism in two out of three trials, while the lean organism was always killed.

Fat and lean tubercle bacilli were exposed to varying concen-

trations of acid fuchsin, from 1:100 to 1:1250. The results for the more dilute concentrations are omitted since the dye failed to kill in any of them. Table 5 records the essential results.

TABLE 3

Results when fat and lean tubercle bacilli were exposed to varying concentrations of phenol

CONDITION OF ORGANISM	TIME EXPOSED	CONCENTRATION OF PHENOL					
		1:100	1:150	1:200	1:250	1:300	1:350
	hours						
Fat	¼	+	+	+	+	+	+
	½	+	+	+	+	+	+
	1	+	+	+	+	+	+
	24	0	0	0	0	0	+
Lean	¼	+	+	+	+	+	+
	½	+	+	+	+	+	+
	1	+	+	+	+	+	+
	24	0	0	0	0	0	+
Fat	¼	+	+	+	+	+	+
	½	+	+	+	+	+	+
	1	+	+	+	+	+	+
	24	0	0	0	0	0	+
Lean	¼	+	+	+	+	+	+
	½	+	+	+	+	+	+
	1	+	+	+	+	+	+
	24	0	0	0	0	0	0
Fat	¼	+	+	+	+	+	+
	½	+	+	+	+	+	+
	1	+	+	+	+	+	+
	24	0	0	0	0	0	+
Lean	¼	0	+	+	+	+	+
	½	0	+	+	+	+	+
	1	0	+	+	+	+	+
	24	0	0	0	0	0	+

The effect of sodium chloride and of ethyl alcohol upon fat and lean tubercle bacilli were next tested. These reagents kill by dehydration rather than by any toxic action. The results with alcohol are given in table 6.

The results showed that 60 and 70 per cent concentrations of alcohol always killed the bacilli and they were therefore omitted from the table. In the first trial, the fat bacilli were killed by 15

TABLE 4

Results when fat and lean tubercle bacilli were exposed to varying concentrations of gentian violet dye

CONDITION OF ORGANISM	TIME EXPOSED	CONCENTRATION OF GENTIAN VIOLET		
		1:5000	1:7500	1:10,000
	hours			
Fat	¼	+	+	+
	½	+	+	+
	1	+	+	+
	24	0	+	+
Lean	¼	+	+	+
	½	+	+	+
	1	+	+	+
	24	0	+	+
Fat	¼	+	+	+
	½	+	+	+
	1	+	+	+
	24	+	+	+
Lean	¼	+	+	+
	½	+	+	+
	1	+	+	+
	24	0	0	+
Fat	¼	+	+	+
	½	+	+	+
	1	+	+	+
	24	+	+	+
Lean	¼	+	+	+
	½	+	+	+
	1	+	+	+
	24	0	+	+

per cent alcohol in twenty-four hours while the lean survived. All the other results were alike for the same concentrations and time.

Sodium chloride, c.p., was tested in concentrations of 8, 15, 20,

25 and 30 per cent. Exposures for one, two and fifteen hours were made in these concentrations. The results are given in table 7.

TABLE 5

Results when fat and lean tubercle bacilli were exposed to varying concentrations of acid fuchsin

CONDITION OF ORGANISM	TIME EXPOSED	CONCENTRATION OF ACID FUCHSIN		
		1:100	1:250	1:500
	hours			
Fat	1	+	+	+
	2	+	+	+
	4	+	+	+
	24	+	+	+
Lean	1	+	+	+
	2	+	+	+
	4	+	+	+
	24	+	+	+
Fat	1	+	+	+
	2	+	+	+
	4	+	+	+
	24	+	+	+
Lean	1	+	+	+
	2	+	+	+
	4	+	+	+
	24	+	+	+
Fat	1	+	+	+
	2	+	+	+
	4	+	+	+
	24	+	+	+
Lean	1	+	+	+
	2	+	+	+
	4	+	+	+
	24	+	+	+

The results do not show any difference in resistance between the fat and the lean bacilli under the conditions employed.

The effect of heat and of ultra-violet light upon fat and lean tubercle bacilli was also tested. The results when the bacilli

were exposed to 60°C., the tubes containing the emulsion being kept in a water bath, are given in table 8.

TABLE 6

Results when fat and lean tubercle bacilli were exposed to varying concentrations of ethyl alcohol

CONDITION OF ORGANISM	TIME EXPOSED	CONCENTRATION OF ALCOHOL			
		50 per cent	40 per cent	30 per cent	15 per cent
	hours				
Fat	¼	0	+	+	+
	½	0	0	+	+
	1	0	0	0	+
	24	0	0	0	0
Lean	¼	0	+	+	+
	½	0	0	+	+
	1	0	0	+	+
	24	0	0	0	+
Fat	¼	+	+	+	+
	½	0	0	+	+
	1	0	0	+	+
	24	0	0	0	+
Lean	¼	0	+	+	+
	½	0	0	+	+
	1	0	0	+	+
	24	0	0	0	+
Fat	¼	0	+	+	+
	½	0	0	+	+
	1	0	0	+	+
	24	0	0	0	+
Lean	¼	0	+	+	+
	½	0	0	+	+
	1	0	0	+	+
	24	0	0	0	+

The results show that in one trial the lean bacilli were killed in sixty minutes, in another the fat were killed under the same conditions, while neither were killed in a third trial.

Lastly, fat and lean tubercle bacilli were exposed to the rays of

TABLE 7

Results when fat and lean tubercle bacilli were exposed to varying concentrations of sodium chloride

CONDITION OF ORGANISM	TIME EXPOSED	CONCENTRATION OF SODIUM CHLORIDE		
		20 per cent	25 per cent	30 per cent
	hours			
Fat	1	+	+	+
	2	+	+	+
	15	+	+	0
Lean	1	+	+	+
	2	+	+	+
	15	+	+	0
Fat	1	+	+	+
	2	+	+	+
	15	+	+	0
Lean	1	+	+	+
	2	+	+	+
	15	+	+	0
Fat	1	+	+	+
	2	+	+	+
	15	+	+	0
Lean	1	+	+	+
	2	+	+	+
	15	+	+	0

TABLE 8

Results when fat and lean tubercle bacilli were exposed to 60°C.

CONDITION OF ORGANISM	TIME EXPOSED AT 60°C.			
	10 minutes	20 minutes	30 minutes	60 minutes
Fat	+	+	+	+
Lean	+	+	+	0
Fat	+	+	+	0
Lean	+	+	+	+
Fat	+	+	+	+
Lean	+	+	+	+

a mercury arc lamp at a distance of 6 inches for different periods of time. The bacilli were emulsified as usual, a loopful of the

TABLE 9

Results when fat and lean tubercle bacilli were exposed to mercury arc rays

CONDITION OF ORGANISM	TIME EXPOSED			
	5 minutes	10 minutes	20 minutes	30 minutes
Fat	+	+	0	0
Lean	+	+	0	0
Fat	+	+	0	0
Lean	+	+	0	0

TABLE 10

Summary of the scores for all the trials, plus indicating merely an advantage in killing power

TABLE	AGENT	TRIAL	BACILLI SHOWING GREATER RESISTANCE		
			Fat	Lean	Neither
II	Corrosive sublimate	1	0	0	+
		2	0	+	0
		3	+	0	0
III	Phenol	1	0	0	+
		2	+	0	0
		3	+	0	0
IV	Gentian violet	1	0	0	+
		2	+	0	0
		3	+	0	0
V	Acid fuchsin	1	0	0	+
		2	0	0	+
		3	0	0	+
VI	Alcohol	1	0	+	0
		2	+	0	0
		3	0	0	+
VII	Salt	1	0	0	+
		2	0	0	+
		3	0	0	+
VIII	Heat	1	+	0	0
		2	0	+	0
		3	0	0	+
IX	Light	1	0	0	+
		2	0	0	+
Totals..	8	23	7	3	13

emulsion placed upon sterile paper slips, exposed to light for varying periods of time, then placed upon nutrient medium for growth. The results of two trials are given in table 9.

The results show no difference between the fat and the lean bacilli in resistance.

If we summarize the results by trials, indicating any advantage in resistance by a plus sign, we have the figures in table 10.

The summary shows that, in a majority of the trials, there was no difference in resistance; in three of the trials the advantage was with the lean bacilli; and in seven it was with the fat. Thus, where a difference occurred between the results for the fat and the lean, the resistance favored the former. It should be noted that whenever a difference in resistance occurred, it was never very marked. Unfortunately it is not possible to ascribe the slight positive advantage showed by the fat bacilli to the fat they contain, since the less favorable medium upon which the fatless strains were grown could account for their decreased resistance. Closer observation seems to indicate that the physical agents and non-poisonous chemicals show no difference, and that when the trials favor the fat bacilli, it occurs with the poisonous chemicals.

DISCUSSION

In 56.4 per cent of the trials no advantage was indicated for either the fat or the lean bacilli; in 30.4 per cent the advantage was with the fat, and in 13.0 per cent it was with the lean, thus giving the advantage to the fat bacilli. A close inspection of the results will show how very slight the advantage was. Only two agents, phenol and gentian violet dye, showed a greater killing power for the lean bacilli. This difference may be due to a slightly decreased vitality of the lean bacilli since they were grown on the presumably less favorable medium, the fat bacilli having the advantage of 5 per cent glycerol.

Obviously the experiments do not completely settle the problem because of our inability to make the two conditions absolutely equal. It would seem that if the greater resistance of the fat bacilli was really due to the lipins they contained, the advantage

should have been considerably greater than was observed. If it is assumed that the lipins do not form an impervious capsule, but that they allow nutrients to permeate the cell protoplasm, as obviously they must for cell growth, then poisons may enter in the same manner. The results are in essential harmony with such an assumption, and seem to indicate that the tubercle bacillus is subject to adverse agents, regardless of its fat content. The fact that the lipins are not digestible in the human body does not affect the general principle involved.

CONCLUSIONS

1. When *Mycobacterium tuberculosis*, H520, apathogenic, was grown upon a nutrient medium containing 5 per cent glycerol it developed approximately 24 to 32 per cent of lipins; on the same medium without glycerol it developed about 5 to 9 per cent of lipins.

2. When the fat and the lean bacilli were exposed to chemicals and to physical agents, more than half of the trials showed no difference in resistance between the two cultures. When a difference was shown, the resistance was slightly greater in case of the fat bacilli.

3. The slightly greater resistance shown by the fat bacilli may be due to their growth on a more favorable medium, containing 5 per cent glycerol.

4. The results do not give a final answer to the question raised, but they tend to show that the so-called fatty capsule does not materially increase the resistance of the bacilli to chemical and physical agents.

The writer is pleased to acknowledge the assistance of Miss Gladys G. Kaiser, Miss Edna Haase, and Mrs. Madeline G. Grahn.

SPECIES OF ESCHERICHIA-AEROBACTER ORGANISMS RESPONSIBLE FOR SOME DEFECTS IN DAIRY PRODUCTS[1]

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Received for publication August 8, 1932

The bacteria of the Escherichia-Aerobacter group have long been recognized as responsible for various defects in dairy products, but comparatively little attention has been given to the identification, on a species basis, of those forms isolated. While there is no immediate prospect that such identification will supply information of importance in connection with the control of the defects, work along this line appears advisable in order to show whether few or many species are involved in the production of abnormalities in milk and its derivatives. The proposals to use the numbers of Escherichia-Aerobacter organisms in pasteurized milk as a means of controlling pasteurizing operations also suggest the advisability of knowing something of the species of this group that may be present in dairy products.

A number of defects in milk and cream, due to organisms of the Escherichia-Aerobacter group, have been studied recently at the Iowa Agricultural Experiment Station and the results of the identification studies are reported herein.

METHODS

Pure cultures of the organisms were isolated from defective milk or cream, or from normal milk from the supply in which the defect was being encountered, by one or more of several methods which may be classified as follows:

I. Direct plating of quantitative dilutions on
   a. Standard agar (for milk analysis).

[1] Journal Paper No. B65 of the Iowa Agricultural Experiment Station.

b. Eosin methylene-blue agar (Levine, 1921).
After incubation at 37°C., cultures were isolated and their purity checked.

II. Enrichment methods.
a. Quantitative dilutions of the sample were inoculated into Durham tubes of Kessler and Swenarton's (1927) gentian-violet lactose peptone bile medium (prepared from the dehydrated product) and incubated at 37°C.
1. After twenty-four hours incubation, material from the lowest and highest dilutions in which gas had been produced was streaked on eosin methylene-blue agar plates.
2. If, after forty-eight hours incubation, gas was present in a higher dilution than after twenty-four hours incubation, material from this tube was streaked on an eosin methylene-blue agar plate.
Cultures were isolated as under I.
b. Enrichment by growing in milk at various temperatures.
1. Several tubes of litmus milk were inoculated with a small quantity of the sample and the inoculated milk incubated at 7° to 10°, 15°, 21°, 32°, and 37°C. As soon as the inoculated milk showed the original defect it was plated in dilution on eosin methylene-blue agar and on standard agar.
2. If the sample was normal when received, it was distributed equally in several sterile, cotton-stoppered, six-ounce bottles and incubated at 7° to 10°, 15°, 21°, 32° and 37°C. As soon as any of the milk showed the defect it was plated in dilution on eosin methylene-blue agar and on standard agar.
Cultures were isolated as under I.

It should be emphasized that enrichment methods may not be equally favorable for all Escherichia-Aerobacter organisms and that, as a result, when more than one species is present their quantitative relationships may be changed.

The cultures isolated were identified according to a modification by Yale (1931) of Bergey's (1930) classification of the genus Escherichia and the genus Aerobacter.

DESCRIPTIONS OF SAMPLES STUDIED

Sample 1 was normal raw milk from a central Iowa supply in which ropiness was being encountered; the standard plate count was 130,000 per milliliter. The milk kept well at 7° to 10°C., but became ropy and developed an unclean, slightly acid odor on standing for a short time at temperatures of 15° to 30°C.

Sample 2 was sour raw cream that was ropy when shipped to

the laboratory from a creamery in northern Iowa, but which lost its ropiness during transit.

Sample 3a was ropy pasteurized milk from a plant in northwestern Iowa. The milk was normal when delivered to a consumer but became ropy on holding in a cellar for forty-eight hours.

Sample 3b was normal pasteurized milk from the plant supplying sample 3a.

Sample 4 was raw milk from a central Iowa supply. It was submitted because a few consumers of the supply complained that the milk seemed to be full of gas. Following its return by a consumer, the sample had been kept in a refrigerator for twenty-four hours before it was brought to the laboratory. When received, the sample appeared normal, but on shaking or warming it foamed markedly. The standard plate count was 590,000,000 per milliliter, and the count on eosin methylene-blue agar (forty-eight-hour incubation at 37°C.) was 510,000,000 per milliliter.

Cottage cheese is made almost daily at the Iowa State College dairy plant from skim milk that has been pasteurized at 145°F. for thirty minutes. As a rule, 1200 pounds of this milk are warmed to about 32°C. in a cheese vat and inoculated with 100 pounds of butter culture. The inoculated milk is kept at about 32°C. until sufficient acid has developed to allow completion of the cheesemaking process.

On October 8, 1931, milk set for cottage cheese developed considerable gas. Sample 5a was taken from the culture used and sample 5b from the gassy milk.

On October 15, 1931, gas formation in milk set for cottage cheese was again encountered. Sample 6a was taken from the culture used and sample 6b from the gassy milk.

On February 18, 1932, milk set for cottage cheese developed acid very slowly (about fourteen hours being required to reach an acidity of 0.4 per cent), and an unclean odor like that produced by some of the Escherichia-Aerobacter organisms appeared. Sample 7 was taken from the milk. The culture used seemed to be satisfactory.

On February 25, 1932, a slow acid development and an unclean

odor were again encountered in milk set for cottage cheese. Sample 8 was taken from the milk. The culture used seemed to be satisfactory.

RESULTS OBTAINED AND COMMENT

The species of Escherichia-Aerobacter organisms isolated from abnormal dairy products in which the defects seemed to be due to organisms of this group, are shown in table 1.

The ropiness which developed in sample 1 on holding in the laboratory was due to *E. neapolitana*, and no other Escherichia-Aerobacter species was found. The culture isolated produced a pronounced ropiness in inoculated milk. Sadler and Middlemass (1926) described a case of ropy milk caused by an atypical *E. neapolitana*.

Sample 2 yielded ropy *A. aerogenes* cultures but no other Escherichia-Aerobacter species. *A. aerogenes* appears to be rather commonly the cause of ropiness in dairy products as is evident from the studies of Buchanan and Hammer (1915), Hammer (1916), Sadler (1917), Mounce (1923) and Yale (1931).

Samples 3a and 3b both contained a variety of Escherichia-Aerobacter species, but only a portion of the isolated cultures produced ropiness in milk. The ropy cultures secured from sample 3a were either *A. aerogenes* or *A. cloacae* while those from sample 3b were *A. cloacae*. Both samples contained *E. pseudocoloides*, which Yale (1931) found to be the most commonly occurring species of the genus Escherichia in pasteurized milk. Yale (1931) also reported that *A. cloacae* was the only species of the genus Aerobacter that he found in pasteurized milk. The variety of species of the Escherichia-Aerobacter group found in the samples suggests rather extensive contamination of the milk.

All of the cultures isolated from sample 4 were *A. oxytocum*. This species appears to be rather common in dairy products. The producer who submitted the sample was told to clean and sterilize his milking machine and other utensils thoroughly. On the day following the clean-up the milk gave a standard plate count of 7000 per milliliter; no colonies were visible on eosin methylene-blue agar plates poured with 0.01 ml. of the milk.

TABLE 1

Species of Escherichia-Aerobacter organisms isolated from samples of defective dairy products

SAMPLE	PRODUCT	DATE RECEIVED	DEFECT	METHODS USED FOR ISOLATIONS	SPECIES ISOLATED
1	Raw milk	6/23/31	Normal when received; became ropy on standing	IIb1; IIb2	*E. neapolitana**
2	Raw cream	8/11/31	Ropy	Ia; IIb1	*A. aerogenes**
3a	Pasteurized milk	10/28/31	Ropy	Ib; IIa1; IIa2; IIb1	*A. aerogenes** *A. cloacae** *A. oxytocum* *E. grünthali* *E. pseudocoloides*
3b	Pasteurized milk	10/28/31	Normal when received; became ropy on standing	Ia; Ib; IIa1; IIa2; IIb1; IIb2	*A. cloacae** *E. leporis* *E. anindolica* *E. gastrica* *E. astheniae* *E. pseudocoloides*
4	Raw milk	9/22/31	Gassy	Ia; Ib; IIa1; IIa2	*A. oxytocum**
5a	Culture for cottage cheese	10/ 8/31	Gassy	Ia; Ib; IIa1; IIa2	*E. coli** *E. anindolica**
5b	Milk for cottage cheese	10/ 8/31	Gassy	Ia; Ib; IIa1; IIa2	*E. coli** *E. anindolica**
6a	Culture for cottage cheese	10/15/31	Gassy	Ia; Ib; IIa1; IIa2	*A. aerogenes* *A. oxytocum* *E. coli* *E. grünthali*
6b	Milk for cottage cheese	10/15/31	Gassy	Ia; Ib; IIa1; IIa2	*A. aerogenes* *A. oxytocum* *E. coli* *E. grünthali*

TABLE 1—*Concluded*

SAMPLE	PRODUCT	DATE RECEIVED	DEFECT	METHODS USED FOR ISOLATIONS	SPECIES ISOLATED
7	Milk for cottage cheese	2/18/32	Slow acid development; unclean odor	Ia; Ib	*A. aerogenes* *A. oxytocum* *E. paragrünthali*
8	Milk for cottage cheese	2/25/32	Slow acid development; unclean odor	Ia; Ib; IIa1; IIa2; IIb1	*A. aerogenes* *E. coli* *E. paragrünthali*

* Definitely responsible for original defect of sample.

Samples 5a and 5b each yielded two Escherichia species, *E. coli* and *E. anindolica*, and these organisms were presumably responsible for the gas formation; the *E. coli* cultures were especially active gas producers. Since the organisms found in the milk set for cottage cheese were also present in the culture used, the difficulty was presumably due to a contaminated culture rather than to the milk.

Samples 6a and 6b represent essentially the same situation as samples 5a and 5b, except that both Aerobacter species (*A. aerogenes* and *A. oxytocum*) and Escherichia species (*E. coli* and *E. grünthali*) were involved. The Aerobacter cultures were very active gas producers.

Samples 5a to 6b, inclusive, represent conditions rather similar to those reported by Hammer (1916).

The milk which developed acid slowly and showed an unclean odor when set for cottage cheese (samples 7 and 8) yielded two Aerobacter species (*A. aerogenes* and *A. oxytocum*) and two Escherichia species (*E. paragrünthali* and *E. coli*). All of the cultures isolated produced an unclean odor when inoculated into milk; the odor produced by the Aerobacter species was more pronounced and objectionable than that produced by the Escherichia species. Cultures of *E. paragrünthali* and *A. oxytocum* which had been isolated from the milk, were studied from the standpoint of their ability to inhibit the development of butter culture organisms. It was found that either of the species or a mixture of the two would inhibit the butter culture organisms if they

were permitted to grow for considerable periods before the latter organisms were inoculated, but would have little, if any, inhibiting influence with short or no preliminary incubation periods. From these observations it seems probable that the Escherichia-Aerobacter organisms were not responsible for the slow acid development in the milk set for cottage cheese.

CONCLUSIONS

A number of Escherichia-Aerobacter species was isolated from dairy products showing defects that were apparently due to organisms of this group. Ropiness was found to be due to *E. neapolitana*, to *A. aerogenes* and to *A. cloacae*. An unusual case of gassiness in market milk was caused by *A. oxytocum*. Gas in butter cultures and in milk set for cottage cheese was apparently due to a variety of Escherichia-Aerobacter species, and the same was true of an unclean odor in milk set for cottage cheese.

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THE GERMICIDAL EFFICIENCY OF SODIUM HYDROXIDE[1]

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Received for publication, Aug. 8, 1932

An increasing need has been felt for an efficient, odorless and relatively non-poisonous disinfectant that would be sufficiently cheap to warrant its routine use in milk houses, dairy barns, swine barns and other farm buildings. No disinfectant in common use fulfills all of these requirements. Some substances are not efficient germicides under actual field conditions and in the presence of organic matter, while certain other disinfectants possess a strong and characteristic odor which makes their use in milk houses and dairy barns impractical. Almost all of the efficient disinfectants are entirely too expensive to justify their routine use under farm conditions and the great majority of the disinfectants are known to be poisonous to livestock when used in their germicidal dilutions.

Preliminary studies on sodium hydroxide indicated a satisfactory germicidal efficiency against non-sporulating microorganisms. High-test commercial household lye was chosen as a satisfactory source of sodium hydroxide.

PREVIOUS WORK ON SODIUM HYDROXIDE AS A DISINFECTANT

As early as 1750 Pringle observed the efficiency of the hydroxides in inhibiting putrefaction and Endemann (1878) reported on work in which bacteria were destroyed by "soda." Inasmuch

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the Graduate School of the University of Wisconsin, June 20, 1932.

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as Endemann commented upon the rapid deterioration of the "soda" solutions following their combination with carbonic acid, there is little doubt that he employed sodium hydroxide as a germicide. Kronig and Paul (1897) referred to the germicidal action of hydroxides and Chick in 1910 reported "the addition to distilled water of sufficient alkali to render the solution N/7000 alkaline increased the mean rate of disinfection at 54°C. about 1.5- to 2-fold."

Thalhimer and Palmer (1911) reported that sodium hydroxide showed marked germicidal activity even in a dilution of 1:2500. They used the Rideal-Walker phenol coefficient method with *B. typhosus* as the test organ sm and reported a phenol coefficient of 23 for sodium hydroxide, while Cooper (1913) found that N/100 sodium hydroxide destroyed *B. typhosus* in forty-five minutes, N/50 in twenty minutes, and N/25 in less than three minutes. These concentrations represent dilutions of sodium hydroxide of 1:2500, 1:1250 and 1:625 respectively.

Halier in 1915 found that sodium hydroxide in concentrations from 0.5 to 1.0 per cent killed anthrax spores in beef, sheep and goat hides within a comparatively short time, and he recommended its use in tanneries. According to work reported in 1916 by Dickinson, the disinfectant power of all soaps is a result of their alkaline reaction. Traube and Somogyi (1921) observed that *B. coli* was less resistant to basic compounds than to acidic substances.

Sierakowski and Milejkowska (1925) reported the degree of alkalinity which they found lethal to several organisms. Their tests led them to conclude that at a temperature of 20°C., *B. coli*, *B. typhosus* and one strain of *B. paratyphosus B* were killed at pH 10.02 while another strain of *B. paratyphosus B* required pH 10.95 for its destruction. *B. paratyphosus A* was killed at pH 10.45 while *Staphylococcus aureus* required the alkalinity represented by pH 12.2 for its destruction.

It becomes apparent that these results indicate a very great germicidal strength for sodium hydroxide, when the theoretical dilutions as well as the low concentrations required to reproduce these degrees of alkalinity experimentally are considered. With

complete ionization of sodium hydroxide and in the absence of either buffer materials or carbon dioxide, pH 10.02 would represent a dilution of sodium hydroxide of almost 1:250,000. However, the lack of complete ionization, the conversion of hydroxide into carbonate, the buffer action of even minute amounts of organic material as well as the ability of the microörganisms themselves to adjust the pH of an unfavorable medium would greatly reduce the actual dilution of the sodium hydroxide represented by pH 10. A pH of 12.2 which was required to kill *Staphylococcus aureus* would theoretically represent a dilution of sodium hydroxide of 1:1666. Experimentally, pH 12.25 at 25°C. was obtained with a 1:600 dilution of commercial lye in the presence of 10 per cent Reddish broth. In the absence of the Reddish broth and at the same temperature, a pH of 12.22 was obtained with a 1:700 dilution.

In 1927, Olitsky and Boez reported that sodium hydroxide was an efficient viricide and that the cheapness of this agent suggested its use as a field disinfectant.

Levine, Buchanan and Lease in 1927 reported the results of the first of a series of experiments upon the germicidal efficiency of the alkalis which led this group of workers to conclude that the undissociated sodium hydroxide rather than the hydroxyl-ion concentration was responsible for the germicidal action of the sodium hydroxide, and in 1928, Meyers published the results of his studies on the effect of the hydroxyl-ion concentration on the thermal death rate of *B. coli*.

Olitsky, Traum and Schoening (1928) found that the virus of foot-and-mouth disease was inactivated by 0.25 to 0.50 per cent solutions of sodium hydrate within three minutes but not within one minute. Incidentally, these workers found that the sodium hydrate could not be satisfactorily replaced by a 5 per cent solution of sodium carbonate, indicating that a certain degree of alkalinity was required for disinfection with alkalies. In tests comparing sodium hydroxide with cresol, they found a 2 per cent solution of sodium hydroxide to be more effective against *Staphylococcus aureus* than was a 3 per cent solution of cresol and that in this concentration, sodium hydroxide destroyed the virus of

foot-and-mouth disease in less than one minute. Under the same conditions, the 3 per cent cresol failed to inactivate the virus in six hours.

Meyers in 1929 published again on the germicidal properties of alkaline washing solutions. He concluded that the pH, the buffer index and the osmotic pressure factor all played a part in the destruction of bacteria by alkalis.

Tilley and Schaffer in 1931 published their results on experimental work in which the germicidal efficiencies of sodium hydroxide, sodium carbonate and trisodium phosphate were compared against *Eberthella typhi*, *Salmonella pullorum*, *Salmonella gallinarum*, *Pasteurella avicida*, *Staphylococcus aureus*, two strains of hemolytic streptococci and spores of *Bacillus anthracis*. They used a strain of *Eberthella typhi* which, at 25°C. was killed by a 1:100 dilution of phenol in seven and one-half minutes but not in five minutes. Under these conditions, sodium hydroxide in a dilution of 1:1400 killed this organism in the same period of time, while a 1:1800 dilution killed in twelve and one-half minutes but not in ten minutes. Other tests were conducted using a strain of *Eberthella typhi* which was more resistent to phenol and which required seven and one-half minutes for its destruction when a 1:95 dilution was used. Sodium hydroxide in a dilution of 1:1200 destroyed this organism under the same conditions in the same period of time. The results obtained with *Eberthella typhi* were fairly representative of those obtained with *Salmonella pullorum* and *Salmonella gallinarum*, while *Pasteurella avicida* was even more susceptible to the action of the sodium hydroxide, being killed by a 1:2000 dilution in seven and one-half minutes. *Staphylococcus aureus* was found to be extremely resistant to the action of most disinfectants. The strain employed required a dilution of 1:70 phenol to destroy it in five minutes, and a 1:120 dilution of sodium hydroxide to kill it in twelve and one-half minutes. Since the germicidal efficiency of phenol solutions appears to bear a ratio of approximately the 5.5 power of the concentration, a 1:70 phenol solution would represent a germicidal strength of approximately seven times that of a 1:100 dilution, while the germicidal efficiency of sodium hydroxide is not propor-

tionally increased by small increases in concentration. In other tests, a 1:150 dilution of sodium hydroxide destroyed *Staphylococcus aureus* between fifteen and thirty minutes, and a 1:200 dilution in less than twenty minutes. Higher concentrations of sodium hydroxide were required to destroy anthrax spores.

McBryde, Niles and Cole (1931) investigated the ability of sodium hydroxide to destroy the virus of hog cholera. They reported that solutions of sodium hydroxide and mixtures of sodium hydroxide and milk of lime were efficient viricides.

EXPERIMENTAL PROCEDURE

Brucella abortus of both bovine and porcine origin, and *Escherichia coli* were chosen as test organisms. Particular attention was paid to the destruction of *Brucella abortus* because of the known infectiveness of the vaginal discharges of infected animals and because of the economic significance of the etiological agent responsible for Bang's disease in cattle and Brucelliasis in other animals.

A high-test commercial household lye[2] was chosen as a satisfactory source of sodium hydroxide. The sodium hydroxide content of the lye was found substantially to exceed the requirements for U. S. P. grade sodium hydroxide. In addition, lye is very cheap and readily available wherever disinfecting need be done.

Several strains of *Brucella abortus* were tested for resistance to both sodium hydroxide and phenol as well as for their ability to initiate growth in Reddish broth within a minimum time. Little consistent difference was noted in the resistance of the various strains to disinfectants (tables 1 and 2) although there appeared to be some parallel between resistance to disinfectants and ability to produce rapid and luxuriant growth. This relationship may be real or only apparent, due to the larger number of organisms in the initial inoculum of the more rapidly growing organism together with the superior ability of the more vigorous strains to initiate growth in subculture. No appreciable differ-

[2] Lewis Lye, supplied by the Pennsylvania Salt Manufacturing Company, was used in these experiments.

TABLE 1

Comparative resistance of five strains of Brucella abortus to 1:800 sodium hydroxide at 25°C.—composite of three trials*

One cubic centimeter of forty-eight-hour *Br. abortus* in Reddish broth in each tube. Ten cubic centimeters of 1:800 sodium hydroxide added.

SUBCULTURES MADE IN	37 BOVINE	93X PORCINE	305 BOVINE	397 BOVINE	PURDUE R BOVINE
minutes					
1	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+++
4	+++	+++	+++	+++	+++
5	+++	+++	+++	+++	+++
6	−−+	+−+	+++	+−+	+++
7½	−−−	−+−	−−−	−−+	−−+
10	−−−	−−−	−−−	−−−	−−−
Minutes to kill.....	6, 6, 7½	7½, 10, 7½	7½, 7½, 7½	7½, 6, 10	7½, 7½, 10

* Actually, 1 cc. of culture was added to 10 cc. of 1:800 sodium hydroxide, as described in the Reddish technic.

TABLE 2

Comparative resistance of five strains of Brucella abortus to 1 per cent phenol at 25°C.—composite of three trials

One cubic centimeter of forty-eight-hour *Br. abortus* in Reddish broth in each tube. Ten cubic centimeters of 1:100 phenol added.

SUBCULTURES MADE IN	37 BOVINE	93X PORCINE	305 BOVINE	397 BOVINE	PURDUE R BOVINE
minutes					
1	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+++
4	+++	+++	+++	+++	+++
5	+++	+++	+++	+++	+++
6	+++	+++	+++	+++	+++
7½	+−+	+++	+++	−++	+++
10	−−+	++−	++−	−−+	+++
12½	−−−	−+−	−−−	−−−	++−
15	−−−	−−−	−−−	−−−	−−−
Minutes to kill.....	10, 7½, 12½	12½, 15, 10	12½, 12½, 10	7½, 10, 12½	15, 15, 12½

ence was noticed in the resistance of *Brucella abortus* of bovine or porcine origin. The Reddish technic, which is essentially the same technic now employed by the United States Food and Drug Administration, was used throughout the experiment.

One-half cubic centimeter of a broth culture of the organism is added to 5 cc. of the disinfectant under test. The broth consists of:

Extract of beef	5 grams
Peptone	10 grams
Sodium chloride	5 grams
Distilled water	1,000 cc.

Especial care was taken to adjust the medium to pH 7, ± 0.1, inasmuch as the germicidal efficiencies of hydroxides were to be tested. Substantial quantities of the medium were prepared at one time and placed in bottles to be used as needed. In this way variations due to slight differences in pH of different batches of media were avoided. Since the laboratory data were intended to be applied directly to the problems of practical disinfection, conditions of actual disinfection were simulated as closely as possible in making the laboratory tests. The sodium hydroxide solutions were prepared on the day they were to be used, but no attempt was made to use carbon dioxide-free water or to prevent the carbon dioxide in the air from coming in contact with the solution.

Suitable water baths were used and the temperature of the solutions was kept constant throughout the period of the test. One cubic centimeter of the culture was added to 10 cc. of the disinfectant solution. Subcultures were taken at regular intervals and incubated at least twice as long as was necessary to obtain luxuriant growth when untreated organisms were inoculated into this medium. Even under these conditions quite wide variations occurred in the time required for the apparent sterilization of the culture as determined by failure of subcultures to grow. It should be remembered, however, that chance may determine whether one less or one more organism than is necessary for the minimum inoculum may be contained in the loopful inoculated into the subculture. While this source of uncertainty cannot be avoided, yet it is almost without practical significance.

It was recognized that with many substances of high antiseptic value, sufficient of the chemical might be carried over with the inoculum to inhibit the growth of the subculture. This is a rather difficult point to eliminate with absolute certainty. Abbott (1891) noted that a concentration of chemical that would not inhibit uninjured cells might effectively inhibit an inoculum of cells partially poisoned by this substance. As a result, the subsequent successful cultivation of the organism in the medium where the treated subcultures failed to grow did not obviate the possibility that the disinfectant may have inhibited the growth of otherwise viable cells. In a series of preliminary tests the results obtained by direct inoculation were compared with the results obtained when efforts were made to further dilute or to neutralize the germicidal agent. Organisms exposed to sodium hydroxide or phenol yielded a greater proportion of growth in direct subcultures than was obtained in parallel subcultures in which the alkali was neutralized or the cells washed free from phenol with physiological saline. Direct inoculation was, therefore, used throughout these tests.

Experiments on the actual disinfection of barns with various agents soon focused attention on the fact that *Br. abortus* was a very unsatisfactory test organism for field work of this nature, because its recovery and rapid identification were difficult. Even in those instances where portions of the floor had been previously inoculated with pure cultures of *Br. abortus*, the ubiquitous members of the colon group quickly outgrew the *Br. abortus* in subcultures. It was, therefore, considered possible that the destruction of the members of the colon group naturally present could be used to indicate the destruction of any abortion organisms that might be present. The resistance of *Escherichia coli* both to phenol and to sodium hydroxide was therefore compared with the resistance of *Br. abortus*. It was found that *E. coli* was slightly more resistant to both disinfectants than was *Br. abortus*. In the subsequent tests carried out under field conditions, *E. coli* was therefore used as a test organism and it was assumed that the destruction of *E. coli* would indicate the destruction of the less resistant *Br. abortus*.

Several different concentrations of commercial sodium hydroxide were experimentally used to disinfect barns. The older directions recommended the use of a 2 per cent commercial sodium hydroxide solution for cleaning the barns prior to disinfection with other substances. Preliminary experiments demonstrated that all the Gram-negative non-sporulating rods were destroyed almost immediately after coming into contact with a 1:150 solution of commercial sodium hydroxide. This concentration was therefore chosen for future work as it was felt that no additional benefit was to be derived from the use of more concentrated solutions. Moreover, the 1:150 solution was only mildly irritating to the skin or mucous membranes of the workers. In fact, it was found that commercial sodium hydroxide solutions of this concentration could be held in the mouth without appreciable injury to the mucous membrane. In addition, the more dilute solution of commercial sodium hydroxide was more economical to prepare. It was thus practicable to use the large volume of disinfectant that is essential to insure all surfaces coming into direct and prolonged contact with the solution.

A definite technic was worked out to test the germicidal efficiency of the commercial sodium hydroxide solutions under field conditions. At various intervals after the application of the commercial sodium hydroxide solutions, sterile swabs were rubbed into the material remaining on the surface of the gutter or manger or on the wall of the barn. These swabs were then transferred into tubes of specially prepared broth to neutralize and dilute the alkali and thus stop its germicidal action. The tubes contained 15 cc. of unneutralized Reddish broth to which had been added a small amount of phenolphthalein.

In no instance was a sufficient degree of alkalinity attained in the medium to cause the phenolphthalein to turn red. It was therefore assumed that the toxic action of the alkali was stopped almost immediately after the swab was placed in the broth. Upon returning to the laboratory, the swabs were transferred to large Durham fermentation tubes containing Reddish broth to which had been added 1 per cent lactose and 1:30,000 crystal violet. This medium had been previously found very favorable

for the growth of even devitalized *E. coli*. These tubes were allowed to incubate for forty-eight hours and discarded as negative at the end of this time if no gas had formed. In case gas was formed, subcultures were made into a second series of a similar medium and incubation continued. Samples from these tubes were Gram-stained and examined microscopically. In no instance were Gram-negative non-sporulating rods recovered from an area that had been disinfected by the 1:150 commercial sodium hydroxide solution. One rather surprising development was the number of Gram-variable and Gram-negative sporulating bacilli isolated from cow barns by this technic. It is surmized that the sodium hydroxide killed the Gram-negative non-sporulating microörganisms, while the crystal violet prevented the germination of the Gram-positive bacilli. It is suggested that this technic might be useful in the recovery of Gram-variable and Gram-negative bacilli.

It was recognized that the actual disinfection of barns and stables always occurs in the presence of considerable quantities of organic material. Comparative tests were therefore made to determine the velocity of disinfection of commercial sodium hydroxide and phenol in the presence of organic matter (table 3). Skim milk was chosen as a uniform source of organic matter that would possess a more than average ability to neutralize the alkalinity of the sodium hydroxide, and thus put this disinfectant under a more severe test. In these experiments, 5 cc. of fresh, autoclaved skim milk were placed in each test tube and allowed to reach the temperature of the water bath. Next, 5 cc. of 1:30, 1:40, 1:50, or 1:60 phenol were added to give a final phenol concentration of 1:60, 1:80, 1:100 or 1:120. In the case of the commercial sodium hydroxide, 5 cc. of a freshly prepared 1:100, 1:150, or 1:200 commercial sodium hydroxide solution were added to the test tubes containing the 5 cc. of milk to give a dilution of 1:200, 1:300 or 1:400. A twenty-four-hour culture of *E. coli* was also placed in the water bath. After an interval of ten to forty minutes, when the different solutions had reached the temperature of the water bath, 1 cc. of the twenty-four-hour culture of *E. coli* in Reddish broth was pipetted into the mixture

of disinfectant and skim milk. Subcultures were taken at various time intervals. Care was taken to hold the temperature of the water bath constant.

Below 30°C. the phenol solutions failed to disinfect within twelve and one-half minutes. In contrast, the commercial sodium hydroxide in a 1:200 dilution killed in five minutes at 1°C., in three and four minutes at 3°C., in ten minutes at 10°C., and in four minutes at 23°C. Above 30°C. both the phenol and

TABLE 3

Velocity of disinfection by commercial sodium hydroxide and phenol in the presence of organic matter

One cubic centimeter of twenty-four-hour *E. coli* in Reddish broth in each tube. Five cubic centimeters skim milk added to each tube. Five cubic centimeters of disinfectant added.

Final Concentration of Disinfectant in Milk-Disinfectant Mixture	1:100 Lye / 1:200 Lye	1:150 Lye / 1:300 Lye	1:200 Lye / 1:400 Lye	1:30 Phenol / 1:60 Phenol	1:40 Phenol / 1:80 Phenol	1:50 Phenol / 1:100 Phenol	1:60 Phenol / 1:120 Phenol
Temperature	Minutes to kill						
°C.							
1	5	12½+	12½+	12½+	12½+	12½+	
3	3	12½+	12½+	12½+	12½+	12½+	
3	4	12½+	12½+	12½+	12½+	12½+	
10	10	12½+	12½+	12½+	12½+	12½+	
23	4	12½+	12½+		12½+	12½+	12½+
30	1	5	12½+		5	12½+	12½+
35	1	5	12½		5	8	10
47	1−	2	3		1−	1	2
50	2	7	8		1	1	4

the commercial sodium hydroxide solutions were efficient disinfectants.

It is well known that certain types of disinfectants soon become inert following contact with organic matter. This would be disadvantageous in the case of a stable disinfectant, as in some instances the disinfectant solution might necessarily remain in intimate contact with organic material before being allowed to come into contact with the organisms to be destroyed. It was, therefore, thought desirable to ascertain whether a rapid loss of

germicidal strength occurred following the admixture of skim milk and commercial sodium hydroxide solutions. Equal parts of skim milk and 1 per cent commercial sodium hydroxide solutions were mixed sixty, forty-five, thirty, fifteen minutes and approximately thirty seconds before the addition of the culture and the taking of subcultures. Only a slight decrease in germicidal efficiency occurred (table 4). Thus in barn disinfection a 1:150 commercial sodium hydroxide solution would retain its ability to destroy microörganisms even after prolonged contact with organic material.

TABLE 4

The rate at which commercial sodium hydroxide solutions decrease in germicidal efficiency in the presence of organic matter

	INTERVAL BETWEEN MIXING OF SKIM MILK AND LYE SOLUTIONS AND THE TEST OF GERMICIDAL EFFICIENCY				
	60 minutes	45 minutes	30 minutes	15 minutes	About 30 seconds
Amount of 1 per cent lye in each tube, cc.	2.5	2.5	2.5	2.5	2.5
Amount of skim milk added in each tube, cc.	2.5	2.5	2.5	2.5	2.5
Concentration of lye in final mixture	1:200	1:200	1:200	1:200	1:200
24-hour *E. coli* in Reddish broth added, cc.	0.5	0.5	0.5	0.5	0.5
Minutes to kill	3	3	3	2	2

Temperature, 14°C.

Laboratory tests were made to determine the time necessary to kill *Br. abortus* and *E. coli* both with sodium hydroxide and phenol at temperatures from 2° and 48°C (tables 5 and 6, figs. 1A and 1B). Using the Reddish technic it was found that at 20°C. the germicidal efficiency of a 1:800 dilution of commercial sodium hydroxide was approximately equal to that of a 1:100 solution of phenol when used against either *Br. abortus* or *E. coli*. It was noted, however, that slight variations in temperature markedly influenced the rate with which the phenol solution killed the microörganisms. In contrast, the velocity of disinfection by sodium hydroxide appeared to be practically independ-

ent of the temperature changes between 2° and 25°C. In this respect the response of the commercial sodium hydroxide solutions to temperature changes differed from that of other disinfectants that have been studied.

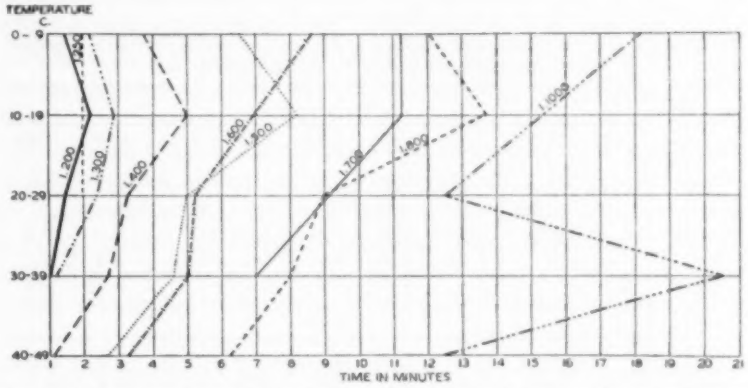

FIG. 1A. THE INFLUENCE OF TEMPERATURE UPON THE TIME AND CONCENTRATION OF COMMERCIAL SODIUM HYDROXIDE NECESSARY TO KILL BR. ABORTUS

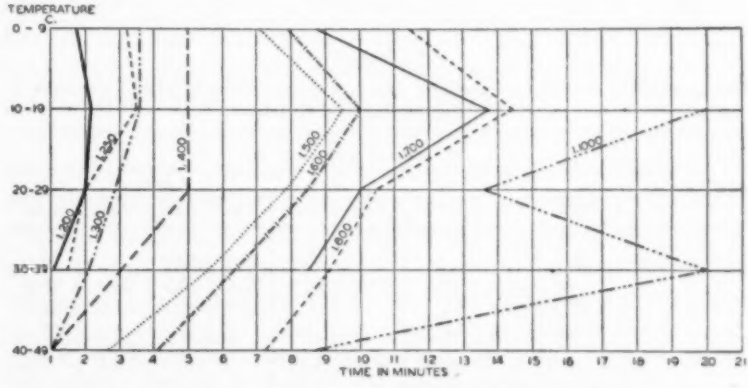

FIG. 1B. THE INFLUENCE OF TEMPERATURE UPON THE TIME AND CONCENTRATION OF COMMERCIAL SODIUM HYDROXIDE NECESSARY TO KILL E. COLI

Since Koch's early work on phenol vapor, it has been recognized that increases in temperature tend to accelerate the velocity of disinfection. Henle (1889) and Behring (1890) referred to the

influence of temperature upon the rate of disinfection, although apparently Heider (1892) was the first to make a systematic series of experiments upon this phenomenon. Madsen and Nyman (1907) found that the increase was about 2.5-fold for a rise of

TABLE 5

The influence of temperature upon the time and concentrations of commercial sodium hydroxide necessary to kill Br. abortus and E. coli

TEMPERATURE	BR. ABORTUS								
	1:200	1:250	1:300	1:400	1:500	1:600	1:700	1:800	1:1000
°C.									
0–9	1.5	2.0	2.2	3.8	6.6	8.75	11.25	12.1	18.3
10–19	2.2	2.0	2.9	5.0	8.2		11.25	13.75	
20–29	1.5	2.0	2.4	3.3	5.0	5.25		9.0	12.5
30–39	1–		1.25	2.7	4.6	5.0	7.0	8.0	20.5
40–49	1–		1–	1.1	2.7	3.3		6.25	12.5
	E. COLI								
0–9	1.75	3.25	3.6	5.0	7.1	7.9	8.75	11.5	20.0
10–19	2.2	3.5	3.6	5.0	9.5	10.0	13.75	14.4	20.0
20–29	2.0	2.0	2.9	5.0	7.85	8.5	10.0	10.5	13.7
30–39	1.1	1.5	2.1	3.1	5.6	6.4	8.5	9.1	20.0
40–49			1–	1–	2.7	4.1		7.25	8.75

TABLE 6

The influence of temperature upon the time and concentration of phenol necessary to kill Br. abortus and E. coli

TEMPERATURE	AVERAGE MINUTES TO KILL									
	Br. abortus					*E. coli*				
	1:60	1:80	1:100	1:110	1:120	1:60	1:80	1:100	1:110	1:120
°C.										
0–9	30+	30+				30+	30+	30+		30+
10–19			30+	30+	30+	30+	30+	30+	30+	30+
20–29		12.5	12.5	25			12.5	11.7	16.6	25
30–39		3	6.5	8.33				5	8.1	15
40–49			1.66	1.66	3.66			2	2.6	3.6

10°C. while Chick (1908) in summarizing her studies on the temperature influence, stated, "The mean reaction velocity of disinfection with metallic salts increases 2- to 4-fold for a rise in temperature of 10°C. In the case of phenol . . . . the temperature coefficient is much higher, usually between 7 and 8."

Experiments to confirm the absence of a marked temperature influence upon the germicidal efficiency of sodium hydroxide solutions were repeated numerous times, with the culture, dilutions and time intervals remaining constant and only the temperature being varied. In each series of tests, it was found that a decrease in temperature from between 25° and 20° to below 10°C. produced no significant decrease in the velocity of disinfection of sodium hydroxide solutions. It was thought that this might be due to the more rapid conversion of the sodium hydroxide to sodium carbonate at the higher temperatures. This possibility was eliminated by two series of experiments. In the first series of experiments, the hydroxyl-ion concentrations of dilute commercial sodium hydroxide solutions were determined over a period of minutes at various temperatures, both when exposed to the atmosphere and when protected from atmospheric carbon dioxide. In neither instance was the immediate decrease in the degree of alkalinity significant. In the second series of experiments, the commercial sodium hydroxide solutions were maintained at temperatures other than those at which the germicidal tests were to be made. The solutions were quickly cooled or heated just prior to the time of the test. The velocity of disinfection by sodium hydroxide solutions remained independent of temperature variations between 2° and 25°C.

It was recognized that this phenomenon was of both practical and theoretical interest. If sodium hydroxide solutions at temperatures below 10°C. were as efficient germicides as identical solutions at 25°C. the laborious and costly heating of water prior to barn disinfection could well be discontinued, and the rapid cooling of the disinfectant while in contact with cold cement floors could be disregarded. Theoretically, the mechanism that caused sodium hydroxide to differ from all other known disinfectants in regard to temperature influence was of great interest. Inasmuch as the activities of the hydroxyl-ions were thought to be responsible for the germicidal efficiency of the sodium hydroxide solutions, a study was made of the influence of temperature changes upon the pH and p(OH) of sodium hydroxide solutions.

THE INFLUENCE OF TEMPERATURE UPON THE pH AND p(OH) OF COMMERCIAL SODIUM HYDROXIDE SOLUTIONS

In order to study the effect of temperature upon sodium hydroxide solutions and similar solutions to which 10 per cent of Reddish broth had been added, electrometric determinations were made of the pH values of the solutions at various temperatures. The pH values were determined from potentiometer readings using the hydrogen electrode. In all instances at least two electrodes were used and in no instance was a reading recorded in which the two electrodes varied as much as 0.0005 volt. The saturated KCl calomel half cell was used with a saturated KCl agar bridge. The saturated calomel half cell was maintained at temperatures varying from 26° to 29° with a temperature of 27°C. in the majority of the readings. The electromotive force of the saturated type of calomel half cell at the temperature at which the half cell was used, was subtracted from the observed electromotive force and the result divided by the absolute temperature of the unknown times the correction factor 0.00019837, according to the formula,

$$pH = \frac{E \text{ observed} - E \text{ cal.}}{0.00019837 \cdot T}$$

as recommended by Sommer. The small junction potential of the KCl agar bridge between the unknown at one temperature and the calomel half cell at another temperature has been ignored.

The pH values of the commercial sodium hydroxide solutions and the commercial sodium hydroxide solutions to which 10 per cent Reddish broth had been added were determined for various temperatures, and graphed against Bjereum's values for pw, the dissociation value of water (figs. 2 and 3). A decreasing value of pH, that is an increase in hydrogen-ion concentration with an increase in temperature should be expected. This is due to the decreasing value of pw as the dissociation of water increases under the influence of a rise in temperature. Actual measurements, however, indicated a rate of decrease of pH value in excess of the rate of decrease of the value of pw. Since pw = pH · p (OH), this would actually indicate a rising value of p(OH), that

is, a decrease in hydroxyl-ion concentration with an increase in temperature (figs. 4 and 5). This phenomenon might be accounted for by the assumption that with a change in temperature

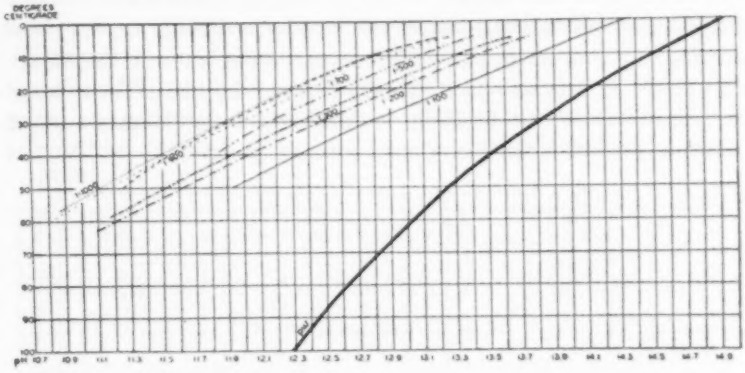

FIG. 2. THE INFLUENCE OF TEMPERATURE UPON THE pH OF VARIOUS COMMERCIAL SODIUM HYDROXIDE SOLUTIONS

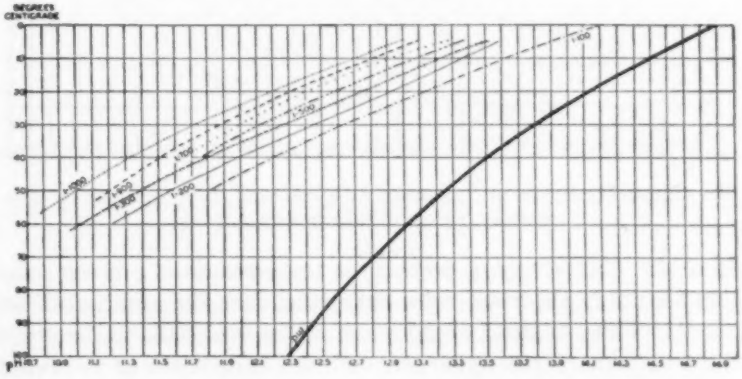

FIG. 3. THE INFLUENCE OF TEMPERATURE UPON THE pH OF VARIOUS COMMERCIAL SODIUM HYDROXIDE SOLUTIONS PLUS 10 PER CENT REDDISH BROTH

the mobility of the hydrogen-ions and hydroxyl-ions may not change at identical rates. Assuming the mobility of the hydrogen-ions to be more markedly increased by a temperature rise

than is the mobility of the hydroxyl-ions, heating would cause an increased ratio of hydrogen-ion to hydroxyl-ion activity.

Further confirmation of the above data is furnished by results obtained from the use of colorimetric pH indicators. Colorimetric pH indicators undergo molecular rearrangement, which results in a change in color, when the balance between the activities of the hydrogen and hydroxyl-ions is altered. When colorimetric indicators are used, an increase in temperature of a given solution produces the same color change as does the addition of

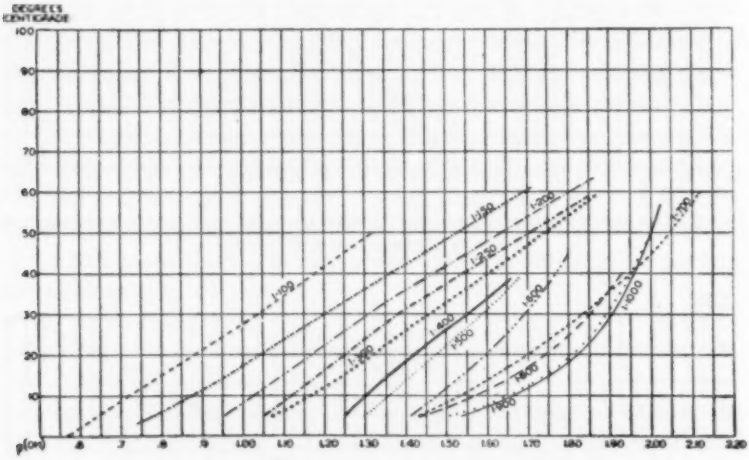

FIG. 4. THE INFLUENCE OF TEMPERATURE UPON THE p(OH) OF VARIOUS COMMERCIAL SODIUM HYDROXIDE SOLUTIONS

an acid. Conversely, a decrease in temperature of a given solution results in the same color change as does the addition of an alkali. Thus, heating would result in the same balance between the activities of the hydrogen and the hydroxyl-ions as would mild acidulation while cooling would have the same effect as would mild alkalinization. With a decrease in temperature the ratio of the hydroxyl-ion activity to the hydrogen-ion activitiy of a given sodium hydroxide solution increases. Cooling from 30° to 2°C. therefore increases the ratio of hydroxyl-ion concentration so that at 2°C. there is approximately two and one-half times the

degree of alkalinity as at 30°C. This represents a decrease in p(OH) of approximately p(OH) 0.4. Therefore, if we assume that the activity of the hydroxyl-ions is responsible for the destruction of bacteria when exposed to sodium hydroxide solutions, it becomes obvious that the decreased ratio of hydroxyl-ion to hydrogen-ion activity approximately balances the increased germicidal effect of each individual hydroxyl-ion at the higher temperature.

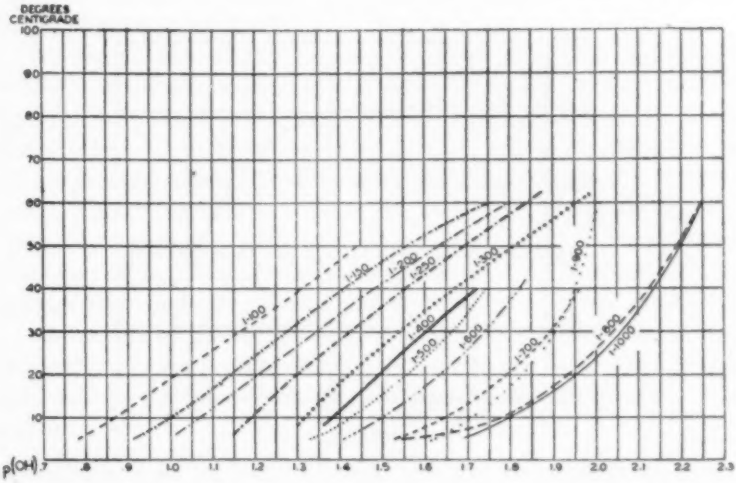

FIG. 5. THE INFLUENCE OF TEMPERATURE UPON THE p(OH) OF VARIOUS COMMERCIAL SODIUM HYDROXIDE SOLUTIONS PLUS 10 PER CENT REDDISH BROTH

Determinations were also made of the pH and p(OH) of sodium hydroxide solutions to which varying quantities of water, urine, and *E. coli* in Reddish broth had been added as it was recognized that commercial sodium hydroxide solutions would be diluted with water and urine as well as mixed with organic material during actual barn disinfection.

The data suggest that the greater ratio of hydroxyl-ion to hydrogen-ion, the lower temperature and the greater germicidal effect of each individual hydroxyl-ion at increased temperatures,

do not balance each other perfectly at all temperatures. Indeed, the germicidal efficiency of a given sodium hydroxide solution appears to be less at temperatures between 10° and 19°C. than between 2° and 9°C. As the temperature approaches 40°C. the influence of temperature becomes apparent. However, even at higher temperatures at which heat is the primary lethal agent, the effect of the decreased hydroxyl-ion concentration still is noticeable. Chick (1910) reported that at 54°C. the addition of dilute alkalis increased the death-rate of *B. typhosus* from 1.5- to 2-fold,

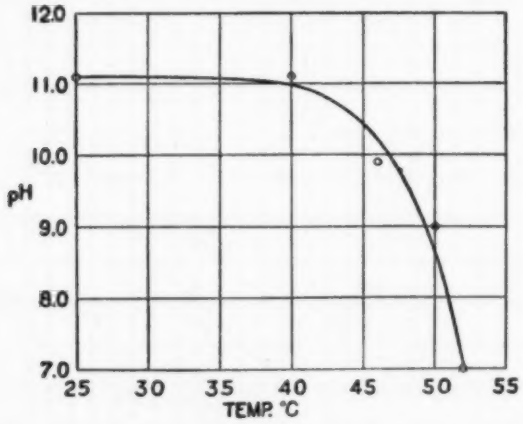

FIG. 6. pH-TEMPERATURE RELATION, SHOWING HYDROXYL ION CONCENTRATION NECESSARY FOR DESTRUCTION OF BACTERIUM COLI AT VARIOUS TEMPERATURES

Time of exposure ten minutes. (After Myers)

while a similar addition of acid increased the death-rate from 5- to 7-fold. Myers (1928) found that temperature changes up to 40°C. did not alter the degree of alkalinity necessary to destroy *B. coli* within ten minutes, although he made no comment upon the reason for this unusual phenomenon (fig. 6).

In a series of publications on the germicidal efficiency of sodium hydroxide and other alkalis, Levine, Buchanan, Lease, Peterson, Toulouse and Lowman have concluded that in alkaline disinfection, the undissociated molecule of sodium hydroxide was more effective than the hydroxyl-ion. The experimental results

obtained by this group of workers vary substantially from those obtained in this work. Lowman, Buchanan and Levine in 1931 found that a 1 per cent solution of sodium hydroxide at 25°C gave a pH of 12.86 with no decrease in alkalinity resulting from fifteen minutes autoclaving at 15 pounds pressure. With the same concentration of sodium hydroxide and at the same temperature, a pH 13.04 was calculated in this work. This decreased to pH 12.90 following autoclaving. The difference between pH 12.86 and 13.04 represents approximately 40 per cent in the degree of alkalinity.

The theory that the germicidal efficiency of sodium hydroxide solutions is largely due to the presence of undissociated sodium hydroxide, appears to be largely based upon the observation that the velocity of disinfection with sodium hydroxide solutions is accelerated by the addition of substantial quantities of a neutral salt. The experimental differentiation of the effects of the hydroxyl-ion and the undissociated sodium hydroxide is difficult. The neutral salts that would suppress the dissociation of the sodium hydroxide might decrease the resistance of the organisms to the action of the hydroxyl-ions, alter the permeability of the cell wall or exert a pronounced influence upon the ability of the bacteria to adjust the medium immediately surrounding the bacterial cell.

It is entirely probable that the actual process of disinfection is the result of all of the above mentioned factors as well as of other diverse influences which were not considered in this work. The interpretation of the data obtained by the above workers is further complicated by the high temperatures employed. The effect of the heat alone should cause the death of some of the bacteria and should greatly lower their resistance to an unfavorable environment.

The concentration of the undissociated sodium hydroxide, like the hydroxyl-ion concentration, would tend to decrease with an increase in temperature. The hydroxyl-ion concentration, however, is directly dependent upon the degree of dissociation of the sodium hydroxide. Any decrease in dissociation of the sodium hydroxide would decrease the hydroxyl-ion concentration. The

experimental data, however, show that the hydroxyl-ion concentration increases with a decrease in temperature. Obviously, the changes in ratio between the hydroxyl-ion and hydrogen-ion concentrations, through a given change in temperature, are much greater than the changes in ratio between the undissociated and dissociated sodium hydroxide. If this were not the case, the hydroxyl-ion concentration of a sodium hydroxide solution would increase with the temperature.

TABLE 7

The velocity of disinfection by commercial sodium hydroxide solutions of the same pH but containing different concentrations of sodium hydroxide

	ORGANIC MATERIAL IN EACH TUBE			
	5 cc. of 10 per cent peptone	5 cc. of 1 per cent peptone	5 cc. of 0.1 per cent peptone	5 cc. of water
E. coli in Reddish broth in each tube, cc.	1	1	1	1
Amount of 1 per cent lye added to bring to pH 11.8, cc.	4	0.9	0.5	0.6
Minutes to kill	25	20	20	20

These pH determinations were made colorimetrically; the actual pH attained was only roughly determined. Since identical color intensities were produced, the pH was probably very nearly equal in the various tubes. The color changes were midway between the yellow and orange color of La Motte Sulfo-orange (pH 11 to 12.6) and red with La Motte Violet, indicating a pH below 12.

Temperature, 14°C.

It would thus appear that the germicidal velocity of sodium hydroxide solutions is primarily influenced by their hydroxyl-ion concentration.

THE GERMICIDAL EFFICIENCY OF SOLUTIONS OF THE SAME p(OH) BUT DIFFERENT SODIUM OR POTASSIUM HYDROXIDE CONCENTRATIONS

Further experiments were conducted to determine whether the hydroxyl-ion concentration alone or the actual amount of sodium hydroxide added to a solution determined the rate of destruction of microörganisms. Peptone solutions of various concentrations up to 10 per cent were alkalinized to the same pH, and their

germicidal efficiencies compared (table 7). La Motte Sulfo-orange (pH 11 to 12.6) was used as an indicator and it was estimated that pH 11.8 was attained. Since the comparative trials were made at the same time, the color produced by the indicator could be compared and nearly identical pH values established. No significant difference was noted in the velocity of disinfection at the same pH values, even though the amount of sodium hydroxide added varied from 4 to 0.5 cc. of a 1 per cent solution. Sodium hydroxide solutions were compared with potassium hydroxide solutions of the same normality and almost the same p(OH) (table 8). Little difference was noted in their

TABLE 8

The velocity of disinfection of E. coli by sodium hydroxide and potassium hydroxide solutions of the same p(OH)

TEMPERATURE	NaOH 1:400 + 10 PER CENT BEDDISH BROTH			KOH 1:286 + 10 PER CENT BEDDISH BROTH		
	Minutes to kill	pH	p(OH)	Minutes to kill	pH	p(OH)
°C.						
3	5, 7	13.52	1.28	6, 5	13.56	1.26
8	6, 6	13.24	1.36	5, 6	13.28	1.32
15	7, 6	12.90	1.44	8, 6	12.90	1.44
20	6	12.68	1.48	7	12.70	1.46
23	5	12.55	1.50	5	12.58	1.47
30	4, 2	12.20	1.62	3, 3	12.22	1.60

germicidal efficiencies. Ammonium hydroxide, even in 50 per cent solution was unable to destroy cultures of *E. coli* within thirty minutes.

CONCLUSIONS

Sodium hydroxide in the form of high-test commercial lye appears to satisfy the need for a disinfectant which is suitable for use in barns and stables, and which is inexpensive, odorless and very efficient against *Brucella abortus* and related micro-organisms.

The velocity of disinfection with sodium hydroxide between 25° and 2°C. is independent of temperature changes. This phenomenon appears to be correlated with the peculiar physico-

chemical reaction of sodium hydroxide solutions to temperature changes, wherein the ratio of the hydroxyl-ion activity to the hydrogen-ion activity increases with a decrease in temperature.

The increase of the hydroxyl-ion activity over the hydrogen-ion activity at the lower temperature approximately balances the greater germicidal efficiency of each hydroxyl-ion at the higher temperature.

At a given temperature, a given hydroxyl-ion concentration possesses a germicidal activity which is independent of the amount of alkali used to attain the hydroxyl-ion concentration.

It is therefore concluded that the activity of the hydroxyl-ions is the most important factor in the destruction of microörganisms by sodium hydroxide.

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OXIDATION-REDUCTION POTENTIALS IN CULTURES OF STAPHYLOCOCCUS AUREUS

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Received for publication, August 18, 1932

In the years since Gillespie (1920) interpreted the potentials developed at a noble metal electrode immersed in a bacterial culture as a reduction potential many papers have appeared in the literature concerning the oxidation-reduction properties of biological systems. Those dealing with the potentials developed in bacterial cultures have been well summarized by Hewitt (1930) who has shown that different organisms under the same experimental conditions develop different but characteristic potentials which do not appear to be dependent alone on the nature of the medium. The studies of Knight and Fildes (1930) indicate that the oxidation-reduction intensity of the medium exerts a decisive influence on the germination of tetanus spores and Allyn and Baldwin (1930, 1932) have likewise demonstrated that the same factor exerts an influence on the ability of certain aerobic bacteria to initiate growth.

The attempts to explain the negative drift of potential observed in bacterial cultures have shown that many factors may be involved in the establishment of these potentials and that these factors are dependent on the specific properties of the system under investigation. Kluyver (1931) has demonstrated that the same bacterial oxido-reduction catalyst is probably capable of acting upon many different substrates and that a single oxido-reduction promoting agent may be responsible for the control of all the oxidations and reductions carried out by a given cell during synthesis and in the maintenance of life. Differences in

the electrostatic properties of these agents might account for the different reactions carried out by various bacterial cells (Quastel, 1928). This may be intimately connected with the oxidation-reduction potentials developed at the surface of the cells and exhibited in the surrounding medium, as shown by the behavior of the reversible oxido-reduction indicators. It is hoped that the results of this investigation may yield a further understanding of this complex problem.

METHODS

Pyrex tubes (4 by 20 cm.), to which were attached two side arms fitted with stopcocks, were employed as culture vessels. One arm, attached to the bottom of the tube and running 15 cm. above the top, served as an inlet for nitrogen. The other arm, resembling an inverted U-tube, was attached to the side of the culture vessel. The latter arm, filled with broth, dipped into a saturated solution of potassium chloride which acted as a salt bridge between the system and the standard saturated potassium chloride-calomel electrode. A film of broth around the central ungreased portion of the stopcock in the side arm made a satisfactory electrical connection and offered no difficulty in maintaining sterile conditions. Two platinum electrodes sealed in soft glass tubing were inserted into each tube through a rubber stopper which also carried a nitrogen outlet tube, the gas escaping under water in a test tube attached by means of a stopper to the outlet. These chambers, filled with the desired media, were sterilized in the autoclave at 20 pounds for one-half hour. All tests were carried out at 37.5°C. in an oil bath.

For studies under anaerobic conditions the small amount of oxygen present in commercial tank nitrogen was removed by passing the gas through a silica tube packed with reduced fine copper turnings maintained at a low red heat and then bubbling the gas through a tall scrubbing tower filled with sodium pyrogallate. Connections to the electrode chambers were made by means of glass tubing and sliding mercury seals. No rubber connections were employed in the purified nitrogen line.

EXPERIMENTAL RESULTS

The value of E_h in Martin's broth (a digest broth prepared from pigs stomachs, pH 7.8) under aerobic conditions is poorly poised in the neighborhood of +0.220 volt. When commercial nitrogen is bubbled through this system at the rate of 50 bubbles per minute a drop in potential is observed, slowly reaching an equilibrium value of approximately +0.075 volt. This results in a lower partial pressure of oxygen in the medium but more constant conditions than those of the ordinary aerobic culture are maintained.

The characteristic fall in potential observed after seeding 75 cc. of broth with 1 drop of a twenty-four-hour culture of *Staphylococcus aureus* (AD-52) is shown in figure 1. Similar results were obtained in smaller vessels under conditions comparable to those prevalent during ordinary aerobic growth in the test tube. However, in the latter case a greater fall in potential was observed during the first few hours, from an original value which was in the neighborhood of +0.220 volt. In about forty hours a potential 20 to 30 millivolts more negative than in the nitrogen-stirred culture was developed. These limiting negative potentials, established in about forty hours, were maintained at a quite constant level for at least four days, after which time the cultures were discarded. During this period the pH slowly shifted from 7.8 to about 8.3. A time-growth curve and a curve representing the fall in potential of sterile broth on deaeration are included in figure 1 for comparative purposes. These curves are based on the average of a large number of tests.

As can be seen from an inspection of these curves the potential falls slowly for the first few hours and then more rapidly during the time in which the organisms are multiplying at a maximum rate. This rate of fall slowly decreases as the number of organisms approaches a maximum and reaches a minimum value about 0.1 volt more negative than the sterile deaerated broth.

In Martin's broth containing 1 per cent glucose the time-potential curve is similar to that of the sugar-free broth, although as low a potential is not reached (−0.03 volt). On standing, the potential increases along with a shift to more acidic conditions.

These results are in general agreement with those reported by Hewitt (1930).

The addition of 0.1 cc. of an antistaphylococcus bacteriophage plus one drop of a broth culture of *Staphylococcus aureus* to the broth resulted in a time-potential drop similar to that of the control culture for the first several hours. However, after about seven hours the potentials increased to a more positive value,

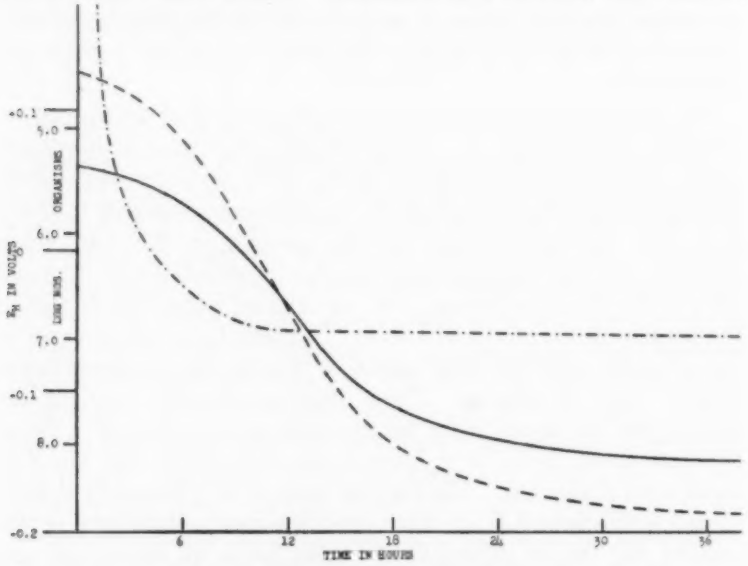

FIG. 1. TIME-POTENTIAL CURVES OF STERILE DEAERATED BROTH (·····) AND CULTURES OF STAPHYLOCOCCUS AUREUS (———) TOGETHER WITH A TIME-GROWTH CURVE (– – – –)

in time usually approaching that of the original broth. These results are also in general agreement with those reported by Hewitt (1931) for aerated cultures of *Staphylococcus aureus* to which bacteriophage had been added. A similar behavior was observed when 'phage was added to the broth three or six hours after the organisms had been added, although a greater time interval was required before the potentials increased to a more positive value. Usually a rather stationary period was noted

about the time lysis occurred as judged by the decrease in the turbidity of the suspension. A second fall in potential was always observed when secondary cultures developed.

When the bacteriophage suspension or the original broth was deaerated with purified nitrogen, approximately the same lower level of E_h, -0.050 to -0.060 volt, was reached in several hours and the potential remained rather constant in this region on longer standing. Deaeration of the sterile filtrate (L 3 Chamberland) of a centrifuged three-day-old broth culture of the organisms resulted in the establishment of an E_h somewhat negative to the above region (-0.06 to -0.07 volt) due to the increased alkalinity of the medium (pH 8.3).

The above results were as a whole visually confirmed by the use of methylene blue and the indigo-sulfonates as oxidation-reduction indicators in the presence of the substrate or of the substrate plus "resting" staphylococci under anaerobic conditions. Such indicators may, however, be misleading at times as they often indicate more intense reducing conditions in the broth than those measured by the electrodes. This discrepancy is probably due to the assumption that the E_h values of these reversible systems are the same in these complex media as in simple buffer solutions.

A negative drift in potential was also observed in sterile broth partially deaerated with nitrogen and then sealed to prevent the diffusion of oxygen into the system as has been reported by Coulter (1928) and others. This system on standing reached an equilibrium value in the neighborhood of E_h -0.05 volt. As recorded above, the passage of commercial nitrogen through the broth poised the potential at a value positive to this, thus confirming the results reported by Knight (1930). These observations indicate that the potential established in sterile anaerobic broth is due to the formation of traces of an active reductant which under aerobic or semianaerobic conditions slowly reacts with molecular oxygen. Under constant conditions the observed potential is controlled by the equilibrium between the rate of formation of this active reductant and the rate of its oxidation.

Clifton and Ort (1930) have shown that the reduction poten-

tials established in deaerated glucose solutions are due to a dynamic equilibrium between a number of molecules temporarily existing in a highly active state and the main bulk of the sugar. This active reductant is almost instantly destroyed by the addition of traces of a mild oxidant; it is constantly replaced and reacts with the oxidant until the latter is completely utilized, at which time a negative drift in potential occurs with the reestablishment of the original reducing intensity. Roepke and Ort (1931) have determined the rate of formation of this active reductant in glucose solutions; the following equation satisfies the experimental observations:

$$\log V = 1.116\ \mathrm{pH} + C$$

were V is the rate of formation of the active reductant and C is a constant. This is in general agreement with the results reported by Wurmser and Geloso (1929) when the differences in experimental conditions are taken into account. Experimental evidence was also advanced which indicated that the active reductant is derived from an ionized form of the sugar molecule.

If a similar mechanism, or mechanisms, is responsible for the establishment of reducing potentials in sterile deaerated broth a constant rate of oxidation should be observed when a suitable oxidant is added to the broth. A solution of 0.075 molar potassium ferricyanide and 0.025 molar potassium ferrocyanide was selected for this purpose. An inspection of the curve in figure 2 representing the addition of 3 cc. of this solution to 75 cc. of sterile deaerated broth shows that the rate of decrease in potential is constant, except for a short interval after the addition of the ferro-ferricyanide at the time marked zero on the graph. During this time equilibrium is being established. A second deviation is observed during the last few minutes when the last trace of ferricyanide is being reduced and this is followed by a rapid negative drift in potential to a value on further standing approximately the same as that of the original broth. This proved always to be the case, even though the amount of the oxidant was varied or when ferricyanide alone was added to the system.

This indicates that a small amount of an active reducing sub-

stance, or substances, is steadily formed from the substrate, and that this reducing substance is oxidized as rapidly as it is formed under the above conditions. Due to the greater intensity of the ferro-ferricyanide system the potentials observed are controlled primarily by this system although those substrate molecules existing temporarily in an activated condition may be responsible for the occurrence of the reaction.

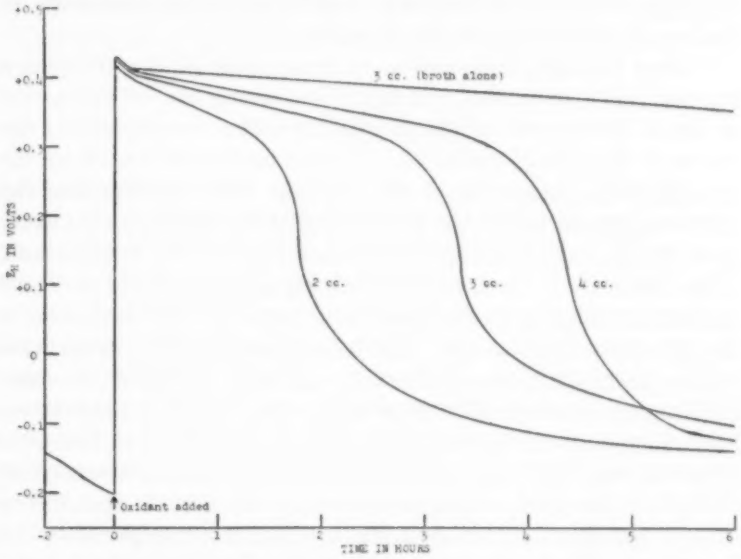

FIG. 2. TIME-POTENTIAL CURVES REPRESENTING THE ADDITION OF A FERRO-FERRICYANIDE SOLUTION TO STERILE DEAERATED BROTH AND TO CULTURES OF STAPHYLOCOCCUS AUREUS

Similar curves are included in figure 2 representing the potential changes following the addition of 2, 3 and 4 cc. of the ferro-ferricyanide solution to a three day old culture of *Staphylococcus aureus*, deaerated for two hours before the addition of the oxidant. It will be noted in these instances that the rate of fall of the potential is more rapid than in broth alone, indicating that the organisms catalyze the reaction, possibly by increasing the concentration of the active form of the substrate molecules. Under

ordinary cultural conditions the rate of activation is probably greater than the rate of oxidation and the equilibrium between the two factors controls the potential of the system. Increasing the partial pressure of oxygen or the addition of ferricyanide or other suitable hydrogen acceptors (dyestuffs) results in a shift of this equilibrium and the development of a more positive potential. This, however, does not mean that the bacterial cells are no longer activating the substrate as before but is a resultant of the two factors which are in operation.

During the two-hour period of deaeration of the cultures a marked fall in potential was noted indicating the accumulation of active reductants in the suspension and a corresponding decrease in the rate of oxidation. This is further indicated by the fact that the intercepts of the straight lines representing the constant rate of fall of the potential (extrapolated to zero time) with the E_h axis lies somewhat below that of the broth alone. This difference is most pronounced when small amounts of the oxidant are employed. This indicates that in the first few minutes the rate of oxidation is quite rapid, decreasing as the accumulated active reductants are oxidized, and finally reaching an equilibrium rate controlled by the rate of activation of the substrate. With larger amounts of added oxidant this effect is less pronounced and the point at which the straight line representing the fall in potential with time intercepts the E_h axis approaches that of the sterile broth as a limit. This point represents the E_h of the medium containing three parts of ferricyanide and one part of the ferrocyanide. The E_h under these conditions is +0.415 volt, a value somewhat below that of the ferro-ferricyanide mixture in a phosphate buffer of the same pH, due to the influence of the salt and other constituents of the broth.

Similar results were obtained when twenty-four-hour old cultures of the organisms were titrated with the ferricyanide or when the titration was carried out under aerobic conditions. This indicates that under the latter conditions the ferricyanide replaces molecular oxygen as a hydrogen or electron "acceptor" in the oxidation of the substrate by the bacterial cells. An increase in potential to a more positive region likewise occurred

when small amounts of hydrogen peroxide were added to the culture. After the hydrogen peroxide was utilized or destroyed the potential slowly dropped to the original negative value.

The rate of fall in potential is also dependent on the concentration of the organisms. For instance, a suspension containing 400,000,000 viable organisms per cubic centimeter reduced 3 cc. of ferricyanide in approximately one-half the time required by a suspension containing 160,000,000 cells per cubic centimeter.

Knowing the value of E_h it is possible to calculate E_o from the well known equation:

$$E_h = E_o - \frac{RT}{nF} \ln \frac{(Red)}{(Ox)}$$

and from this equation calculate the concentration of the ferricyanide at any time directly from the potential readings. The value of E_o for the ferro-ferricyanide system is independent of the pH in the region studied.

The values for the amount of ferricyanide present at various intervals of time were calculated from the data plotted in figure 2 and are plotted in figure 3. The rate at which the ferricyanide was reduced by the broth alone was 0.00037 of a millimol per minute while in the presence of approximately the same number of organisms this rate varied from 0.0014 to 0.0016 of a millimol per minute. Under the same conditions about 0.0004 of a millimol of ferricyanide was reduced per minute in a 'phage lysed culture or in the filtrate (L 3 Chamberland) of either a three- or six-day old culture. If side reactions were involved the rate of reduction should decrease with the concentration of the oxidant but since the ferricyanide is removed at a constant rate throughout a wide range of its concentration it must be oxidizing practically instantaneously activated reductants formed at a constant rate.

Knowing the number of viable organisms in the broth it is possible to calculate the amount of ferricyanide reduced per cell per minute. Approximately 0.0015 of a millimol of ferricyanide was reduced per minute in the presence of 2.33×10^{10} cells and therefore 6.6×10^{-14} millimols or 4×10^{7} molecules were reduced

per cell per minute. This is of the same order of magnitude as other reductions carried out by bacteria.

Since the average rate of reduction of the ferricyanide in the filtrate of a broth culture of staphylococci is not much greater than in broth alone it is evident that no appreciable amount of reducing substances is liberated by the cells. As another control 1×10^{10} staphylococci were suspended in 15 cc. of distilled water and held for one month at 37.5°C. This suspension of old,

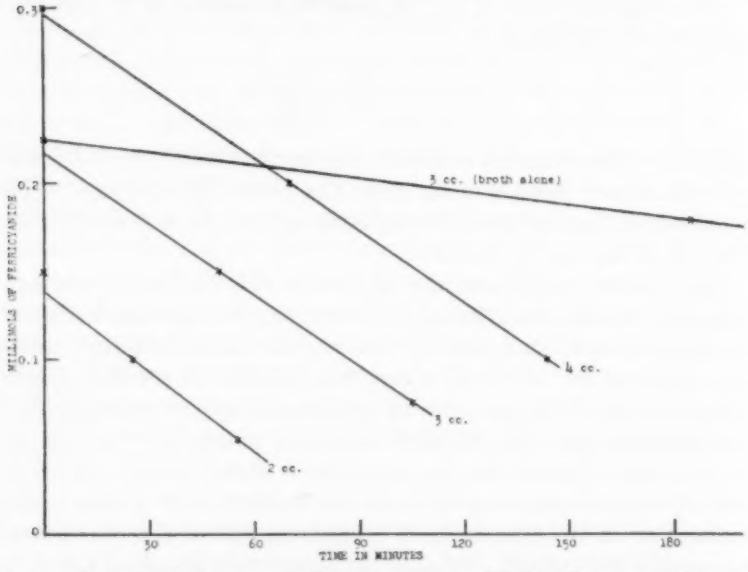

FIG. 3. RATE OF REDUCTION OF FERRICYANIDE BY STERILE DEAERATED BROTH AND CULTURES OF STAPHYLOCOCCUS IS CONSTANT

partially autolysed, cells was added to 60 cc. of broth, deaerated for two hours, and titrated with 3 cc. of ferro-ferricyanide. Approximately 0.0005 of a millimol of ferricyanide was reduced per minute in this test, a value not much greater than that observed in broth alone.

Also a three day old culture of staphylococci, was held in boiling water for fifteen minutes, cooled to 37.5°C., and then tested as above. A marked rate of fall in potential was observed

for the first twenty minutes, after which an equilibrium rate of reduction of 0.0007 of a millimol per minute was established. This somewhat higher value may be due to a residual enzymatic activity of the cells or to a change in the chemical composition of the medium.

DISCUSSION

Michaelis (1930) states "the redox potential of a cell suspension is the redox potential of the medium in which the cells are suspended." Coulter and Isaacs (1929) also advance the same general conception and state that "the bacteria (*B. typhosus*) influence the potentials in the first period of their growth by exhaustion of oxygen from the culture, thus permitting the characteristic potential of the culture medium to become manifest." A further decline in potential after the maximum growth period to values more negative than those of the culture medium suggested to the authors the liberation of reductive substances by the dissolution of the organisms. Hewitt (1931) on the other hand suggests that the potentials developed in bacterial cultures are due mainly to the metabolic activities of the cells.

Bearing in mind that the observed electrode potentials are measured against the liquid in which the cells are suspended it seems more reasonable to assume that the observed potentials are a measurement of the intensity of the oxidation-reduction reactions carried out at the cell surface and in the medium. Evidence has been presented in this communication that the observed potentials in sterile deaerated broth are due to a dynamic equilibrium between the normal molecules and a few existing temporarily at a higher energy level and in an electromotively active state. The potentials observed in cultures of *Staphylococcus aureus* are then due to a shift in this equilibrium with the establishment of a new equilibrium between the rate of activation of the substrate molecules of the medium by the cellular catalytic agents and the rate at which they are oxidized. When oxidizing or reducing substances are formed by the organisms a shift in potential may occur as is shown by the behavior of electrodes in cultures of organisms liberating hydrogen or hydrogen

peroxide. Boyd and Reed (1931) have advanced evidence that there is a non-gaseous electromotively active system in cultures of *E. coli* in addition to the gaseous system developed during oxidative decomposition of glucose.

A suspension of well washed and aerated "resting" staphylococci *in vacuo* will not reduce appropriate redox indicators but when a suitable hydrogen donator is added to the suspension a reduction of the dye will occur. This is likewise true for other organisms and indicates that no appreciable amounts of reducing substances are liberated by the cells. Different potentials are observed in cultures of different types of bacteria in the same medium and these observations also lend support to Hewitt's hypothesis. The observations reported in this paper favor this hypothesis and indicate that the substrate molecules are activated at a constant rate by the organisms and that the observed potentials are a resultant of the rate of activation and of oxidation. Plotz and Geloso (1930) have shown that the addition of platinum black to sterile broth *in vacuo* results in time-potential shifts analogous to those observed in cultures of certain anaerobes. This was interpreted as signifying that the bacteria, like the platinum black, acted as a catalyst. The measured potentials are a resultant of the chemical and physical activities at the surface of the catalyst. Therefore the potentials observed in bacterial cultures can likewise be considered as a measurement of the intensity of the bacterio-catalytic decomposition of the substrate. Other bacteria may activate the substrate molecules to form other types of "active" reductants which may exert a different potential or the oxidation may follow a different course at a different intensity level. This might be due to the establishment of a different equilibrium between the rates of activation and oxidation with a subsequent shift in potential. This activation may be in the sense postulated by Quastel with the resulting tendency of the molecule to act as a hydrogen—or electron—donator. The potentials observed in the anaerobic suspensions of bacteria may then well yield a measurement of the "electron-escaping tendency" of the activated molecules. Needless to say many other factors may play a rôle in the estab-

lishment of the potentials in these complex systems but the hypothesis that the observed potentials are a resultant of the metabolic activities of the cells in the medium seems to the author to be in closest agreement with the modern conceptions of bacterial metabolism.

SUMMARY AND CONCLUSIONS

Cultures of *Staphylococcus aureus* in broth develop and maintain rather intense reducing conditions.

Following lysis of the organisms by anti-staphylococcus bacteriophage the potential increases to a more positive value.

Evidence is presented that there is a dynamic equilibrium between the rate of formation of "active" reductants in sterile deaerated broth and the bulk of the molecules in their normal state. The presence of an oxidizing agent results in the establishment of a more positive potential, controlled by the intensity of the added system and the rate of oxidation-reduction.

Cultures of *Staphylococcus aureus* reduce potassium ferricyanide at a constant rate under either aerobic or anaerobic conditions.

Data are presented which suggest that the observed oxidation-reduction potentials in bacterial cultures are a resultant of the metabolic activities of the cells.

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STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

III. THE DIPHTHERIA BACILLUS

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Received for publication August 14, 1932

INTRODUCTION

It appears from many investigations of the requirements for growth of certain of the pathogenic bacteria, that a most complex metabolic situation is to be dealt with. Thus, with the exception of an occasional organism occurring naturally in the animal body which grows readily on the simplest of the so-called "synthetic media,"—including the colon bacillus, some of the staphylococci and, curiously enough, the tubercle bacillus together with a few others,—it has been impossible to prepare media of known composition upon which the remainder would proliferate normally.

It is perhaps aside from the point to consider in great detail the various attempts that have been made to accustom or acclimate a given species of organism to a synthetic medium by the gradual withdrawal of a suitable menstruum and the substitution of a solution of known composition. In view of the present status of the question of bacterial dissociation, it is quite impossible to predict what may take place in the alterations of metabolism in the bacterial cell subjected over long periods of time to gradually changing conditions, or to draw sweeping conclusions as to the relation between the initial and final stages of such cultures from a biochemical standpoint. In certain specific problems, perhaps such a method of approach may lead to fruitful results, but it avoids directly the primary problem of the nature of the substances which most pathogenic bacteria seem to require for their growth.

Without speculation as to the possible applications to bacteriological methods which might develop from a more exact knowledge of this sort, there is the probability that many facts of general biological importance would result. The various types of pathogenic bacteria under consideration, including some staphylococci, streptococci, and pneumococci, the diphtheria bacillus, the meningococcus and gonococcus, the influenza bacillus, as well as others less well studied, grow naturally within or upon the animal body, in the presence of an exceedingly complex mixture of substances. In the test tube, they flourish progressively better as the medium is enriched by more and more complex products, serum, whole tissue, blood, etc. Repeated efforts to substitute the known chemical components of the animal body have either failed or been only partially successful. One is therefore forced to the conclusion that substances of still unknown composition, or of unrecognized importance are to be found in the body, a knowledge of which would be as important to the biochemist and physiologist as to the bacteriologist, if from perhaps different points of view.

The view is generally held among bacteriologists that certain of the so-called "enriching" substances, such as blood or serum, owe their influence to the presence of native proteins, and that in certain instances (gonococcus) even the species of animal from which the protein is derived may be important. There is also the possibility that tissue enzymes may be directly involved, or that certain colloidal or perhaps surface-tension conditions of the body fluids are difficult to duplicate in artificial mixtures. While such considerations indicate the complexity of the problems involved, there is abundant evidence that a number of comparatively simple, or at least relatively stable chemical constituents also play an important rôle. In most cases where an attempt has been made, chemically, to define the nature of growth requirements among these various organisms, the results have been sufficiently encouraging to warrant a continuation, while at the same time showing very clearly the practical difficulties. Fractions obtained in certain instances have been effective in such great dilution that the "vitamine" conception has been definitely suggested, with all the uncertainties which have developed from many years'

investigation of these substances in animal nutrition. Until the chemical nature of the vitamines, as well as that of the various substances involved in bacterial nutrition is much better understood than at present, it is obviously not possible to know definitely what the relation between them may be. In any case, bacterial growth as a test is infinitely more practicable than animal feeding experiments, and, while obviously less satisfactory than chemical analysis, approaches it in rapidity.

With the possibility in mind that one or more of these fairly stable "accessory factors" might be required in common by a number of organisms which normally inhabit the animal body, and which fail to flourish on media of known composition, and that additional factors may be necessary only in the case of certain more fastidious organisms, one of the writers undertook, some years ago, a study of the requirements of the hemolytic streptococci and pneumococci (Mueller, 1922). A certain amount of information was obtained in this work, but the requirements proved to be fairly complex, and it has been felt that further work might well be done with organisms thriving on simpler systems. Consequently, Hughes (1932), in this laboratory, undertook a study of the staphylococci in which it was shown that but a single accessory factor was probably involved. This substance was not isolated, however, and work upon it should be continued. In the present paper we wish to describe the results of experiments with the growth of the diphtheria bacillus.

It has seemed to us logical to attack these problems from the angle of attempting to determine the nature of the substances present in a medium which is suitable for growth of a specific organism, rather than to start with a basic solution of known composition, adding—successively—different substances in hopes of finding a satisfactory adjuvent more or less by accident. That the former plan is more likely to prove successful, in spite of the labor involved, is indicated in the two reports referred to above, as well as in the work to be here described. There is fairly definite evidence that in each case substances of unknown composition, or of unrecognized importance, are involved which could scarcely be encountered accidentally. That such substances should be

capable of isolation and identification is evidenced by the fact that a quite satisfactory degree of chemical stability has been shown by the substances sought.

THE DIPHTHERIA BACILLUS

A great deal of work has been already reported on the growth of the diphtheria bacillus in synthetic media. This need not here be reviewed in detail, but reference may be made to the work of Maver (1930) in which the literature is summarized. For the most part, the work has consisted in the addition of such substances as amino acids to inorganic salt mixtures, and in the endeavour to accustom strains of the organism to such media by serial transfer. Curiously enough, an occasional strain of the organism is able to proliferate on such media, and even to produce a weak toxin. In the main, however, good growth of the majority of strains is obtained only on "semi-synthetic media," such as that described by Wadsworth and Wheeler (1928), which contains commercial "Proteose-Peptone." The presence of this substance of course removes such media completely from the field of solutions of known composition. The chief interest of this work lies in the fact that potent toxins appear to be produced on media containing no muscle extractives. It should, however, be pointed out that the source of "Proteose-Peptone" is not commonly known, nor its method of preparation. If tissue protein is used, obviously extractives are actually present, and it will be shown later that the extractive required for the growth of certain strains of diphtheria bacillus is a sufficiently stable substance to withstand any treatment probably used in the commercial preparation of the peptone. Moreover, we have found that "Proteose-Peptone" appears to contain small amounts of creatine, a substance present in tissues and ordinarily not encountered elsewhere. We are therefore inclined to the belief that media prepared with "Proteose-Peptone" differ only from the usual infusion medium in being, not free from extractives, but low in extractives. That most culture media are exceedingly wasteful in containing a far higher percentage of extractives than necessary has been shown not only in the work with streptococcus already

referred to (Mueller, 1922) but by experience of others (Davis and Ferry, 1919) as well as ourselves with the diphtheria bacillus and by practical experience with the streptococcus (Mueller and Klise, 1932) and other organisms.

It does not seem essential to include in this report lengthy protocols of the numerous individual experiments which have been carried out, nor to describe in great detail the chemical manipulations to which the various fractions have been subjected. It has been possible to identify certain of the substances shown to be necessary for the strain with which we have worked, with compounds already known, whereas the nature of other fractions still remains obscure, and their investigation is being continued.

A strain of diphtheria bacillus isolated by Yü (1930) in this laboratory, and shown by him to be of smooth colony type and a strong toxin producer, has been used throughout this study. A comparison of its requirements with those of several other strains has shown that its needs are rather more complex than those of some of the others, but this was not recognized at the beginning of the work. It has been possible, however, to work out a series of controls in which there appears to be only one variable for each factor studied.

This strain grows well, with rapid pellicle formation, on infusion broth containing proteose peptone and has been carried through the period covered by these experiments by daily transfer on such broth. Its ability to produce toxin has not been re-tested, but the growth requirements have apparently not changed in the case of any of the essential factors to be described.

It was shown (*a*) that a sulfuric acid hydrolysate of casein to which tryptophane (which is destroyed by acid hydrolysis) was added, could be substituted for proteose peptone, and (*b*) that Liebig's meat extract could be substituted in this latter medium for an infusion of fresh meat. A mixture of inorganic salts was used in such media, and our solution which was to be investigated had the following composition:

	per cent
Protein hydrolysate	1.0
Liebig's extract	0.5
Tryptophane	0.01

	per cent
$NaCl$	0.5
K_2HPO_4	0.1
$CaCl_2$	0.01
$MgSO_4$	0.02
$FeCl_3$	Trace

The separation of a mixture containing, for the most part, amino acids obviously offers fewer difficulties then would that of a complex mixture of peptones, polypeptides and amino acids. Further, the use of Liebig's extract instead of a fresh meat infusion, while perhaps not a simplification, at least provides a ready supply of initial material of fairly constant composition, particularly if a single large lot is used for many experiments. Growth will take place neither on the salt mixture and Liebig's extract alone, nor on the former plus the casein hydrolysate and tryptophane. It is therefore possible to vary each of the complex components separately, holding the other constant.

NECESSITY FOR TRYPTOPHANE

Casein hydrolysate added to the inorganic constituents and Liebig's extract proved unable to support growth of the strain of diphtheria bacillus used. Since, however, tryptophane is destroyed by acid hydrolysis, and is the only known amino acid which fails to survive such treatment, small amounts of this substance were added to the medium and it was found that satisfactory growth was at once obtained. The smallest quantity which appeared to give optimal growth was about 0.001 gram in 10 cc. of the medium, and this quantity has consequently been added in subsequent work. The fact that tryptophane enhances growth of this organism under certain circumstances has been brought out by other workers, but it is of interest that the Liebig's extract should contain an insufficient amount of the substance. In the present system, therefore, tryptophane represents the first known essential factor.

NATURE OF NECESSARY FACTORS OF CASEIN HYDROLYSATE

The mixture of amino acids obtained by eighteen to twenty hours' hydrolysis of casein with 30 per cent sulfuric acid is freed

from excess H_2SO_4 with $Ba(OH)_2$, and concentrated to a small bulk *in vacuo*. It is then subjected to continuous extraction with butyl alcohol, *in vacuo*, according to the method of Dakin (1920). Three fractions are obtained by this method; (*a*) the monoamino acids which separate as a grey or white powder from the butyl alcohol; (*b*) the butyl alcohol solution which contains proline, some polypeptid anhydrides and perhaps other substances, and (*c*) the material remaining in the aqueous solution, not extracted by butyl alcohol, consisting of the basic amino acids and the dicarboxylic acids.

Tests carried out by adding appropriate amounts of these three fractions to the basic solution with Liebig's extract showed that only fractions (*b*) and (*c*) appeared to be essential, fraction (*a*) exerting no evident influence.

Investigation of fraction (*b*). This material, freed from butyl alcohol and small amounts of water-insoluble material, contains mainly proline. In view of the characteristic chemical nature of this substance, it was presumed that the activity of the fraction depended upon its presence. Following the further directions of Dakin for the purification of proline from this source, we prepared a moderately pure substance, which, however, owing to its extreme solubility, was almost surely not completely pure. This substance, while still permitting some growth when added to media, appeared to be less effective than the original crude fraction. In an endeavour to approach the problem from a different point, synthetic proline was prepared by the method of Sörensen and Anderson (1908). The product obtained here also was probably not completely pure, but at any rate it was unlikely that the same impurities present in the protein fraction would be encountered. This material, consisting of course of the racemic acid, was completely inactive in promoting growth, but was not directly inhibitory, i.e., the additon of both the synthetic substance and fraction (*b*) above yielded satisfactory growth.

A further investigation of this fraction is in progress. At present, one can only conclude that proline alone is probably not the required material, although it may well be that proline plus a second material will be found to be essential.

Investigation of fraction (*c*). The amino acids recognized as composing this fraction—arginine, histidine, lysine, glutamic, aspartic and hydroxy-glutamic acid—were substituted for it singly and in combination, but no growth was obtained. It was finally found that cystine, the presence of which in the material had not been recognized, could take the place of the mixture, and it appears to yield just as abundant growth as the total fraction (*c*). The amount required for maximum growth is, as in the case of tryptophane, about 1 mgm. per 10 cc. of medium, added as the hydrochloride. We have attempted unsuccessfully to isolate cystine from the crude fraction. It is probably present in small amounts, for colorimetric tests distinctive of cystine have been obtained from certain partially purified preparations. It has not been shown, however, that cystine is the only substance in this fraction which plays a part in rendering the medium suitable.

NATURE OF ESSENTIAL SUBSTANCE IN LIEBIG'S EXTRACT

In carrying out experiments in which fractions of Liebig's extract are to be tested, the basic salt mixture plus 1 per cent of edestin hydrolysate has been used, instead of casein hydrolysate. This is for the reason that frequently, in the use of the products from casein, control tubes—i.e., salt mixture and casein hydrolysate—show slight growth. By using a preparation from edestin, such is never the case, control tubes remaining completely negative.

The Liebig's extract has been subjected to various types of separation. The essential material is dialyzable, but so is a very large proportion of the inert material. It is fairly stable to heat, withstanding autoclaving in neutral or moderately acid reaction. In the presence of alkali it is less stable when heated. It is not precipitated by tannic acid, lead acetate, picric acid, phosphotungstic acid, nor by silver salts under any conditions that have been tried. Evidently, then, it is not a basic substance. Briefly, the most satisfactory method so far worked out for its partial purification is the following:

The Liebig's extract is dissolved in warm water, using one liter of water to a pound of the substance. Solid picric acid in

excess is added (about 500 grams), and after very thorough stirring and grinding in a large mortar, it is set in the cold room over night. The precipitate is then filtered off and washed with ice water. The combined filtrate and washings are strongly acidified with H_2SO_4 until no more precipitate (mostly picric acid) separates, and this is removed. Ag_2O is then added in excess, and the fractions of material precipitable by silver in acid, neutral and alkaline reaction are successively removed. The solution is reacidified with H_2SO_4 and precipitated by posphotungstic acid after concentration *in vacuo*. The precipitate is removed and the still acid solution is extracted continuously with ether for two days, removing phosphotungstic acid, picric acid and lactic acid. It is then concentrated *in vacuo*, and, still acidified with H_2SO_4, is precipitated with three volumes of ethyl alcohol. This separates a considerable quantity of sodium sulphate, together with perhaps small amounts of the sulphates of other metals which may have been present. From this point, no suitable procedure has been devised. A variable amount of inert substance can be removed by crystallizing from fairly strong alcohol in concentrated solution. The only substance identified which can be removed at this stage is o-pyrrolidone carboxylic acid. The alcoholic filtrates and mother liquors retain the active fraction, which is extremely soluble both in water and alcohol, and probably is by no means uncontaminated with inert material. From a pound of Liebig's extract one obtains only a few tenths of a gram of a practically colorless syrup, which shows no tendency to crystallize on long standing in the desiccator. No reagent has been found which will precipitate the active material, and attempts to crystallize it from mixtures of alcohol, acetone, methyl alcohol, ether, etc., have been unsuccessful.

A fraction of a milligram of this material, in 10 cc. of the saltedestin hydrolysate mixture, is sufficient to insure growth. The amount of pellicle formed is not as great as that obtained by the use of the entire Liebig's extract, but it appears to be the one fraction which is absolutely essential to obtain any growth. It is obvious that the method here described is laborious and rather costly, and in continuing the investigation of this fraction it may

be possible to find a more suitable source with relatively less and perhaps fewer inert materials.

SUMMARY

Starting with the usual laboratory infusion-peptone broth, supporting good growth of the strain of diphtheria bacillus under investigation, the organic components have been simplified and separated as far as possible in order to determine what chemical substances in such media are actually required by this organism. It is not to be supposed that these materials, if combined alone, would form a complete medium. Naturally sources of nitrogen and carbon must be present, and it is very probable that here, as in the case of other organisms, a variety of materials will serve the purpose. In our experiments, while testing fractions of amino acids, 0.5 per cent Liebig's extract, rich in nitrogenous material of all sorts, is supplied, and conversely, in testing fractions of the latter, approximately 1 per cent of amino acid mixture is present. An abundant supply of nitrogenous material has therefore always been available.

It seems probable that these fractions represent characteristic chemical groupings which the diphtheria bacillus, being accustomed to growth only in the presence of highly complex organic substances, has never developed the ability to build up out of simpler materials. This is obviously the case with the two fractions, tryptophane and cystine, which have been shown to be essential.

The following diagram will perhaps represent the separation as it has been carried out:

Meat infusion-peptone broth	Salts	Salts	Salts
	Peptone	(1) Amino acid mixture resulting from H_2SO_4 hydrolysis of protein	(1 a) Cystine
			(1 b) An unidentified alcohol soluble substance
		(2) Tryptophane	(2) Tryptophane
	Meat infusion	Liebig's extract	An unidentified non-basic substance

CONCLUSIONS

In addition to tryptophane and cystine, two so far unidentified substances appear to be essential for the growth of a strain of diphtheria bacillus. One of these is associated with proline in the Dakin butyl alcohol extraction method of amino acids, but evidently is not proline itself. The other, obtained from Liebig's meat extract, is non-basic and not precipitable by any of the usual precipitants. It is highly soluble in both water and alcohol. Further work is being carried out on these two fractions.

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THE ACTION OF HEXYLRESORCINOL ON BACTERIA IN CERTAIN FOOD PRODUCTS

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Received for publication September 1, 1932

The number of chemicals employed as food preservatives is somewhat limited. The Food and Drugs Act permits a few of these to be used in food products. Owing to the fact, however, that there is no uniformity of opinion as to the absolute harmlessness of these food preservatives, any new preservative which can be shown to be harmless would have a distinct advantage. It was thought that hexylresorcinol might be used as an efficient food preservative, since this substance has a very high phenol coefficient.

Johnson and Lane (1921) in studying alkyl resorcinols found the phenol coefficient to increase as the series ascended. Leonard and Feirer (1927) reported that the phenol coefficient of hexylresorcinol was around 70. Leonard (1924) determined that, coincident with the sharp rise in bactericidal power with an increase in the length of the side chain, there was a corresponding drop in toxicity. Leonard (1924) and Leonard and Wood (1925) concluded from their experiments that hexylresorcinol was without deleterious effect on rabbits and human beings. Experiments have been conducted in this laboratory on the effect of hexylresorcinol on the growth, reproduction, and lactation of the white rat and will be reported in a separate communication.

A number of representative foods were selected to be tested with hexylresorcinol. Equal weights of each of the raw test foods were placed in sterile flasks and varying amounts of hexylresorcinol[1] of known concentrations were added to the different

[1] The hexylresorcinol used in this research was kindly furnished by Dr. G. H. Krall, Sharpe and Dohme, Baltimore, Maryland.

flasks, one flask of the food, without the hexylresorcinol, being kept as a control. A very concentrated solution of hexylresorcinol was prepared using 50 per cent glycerol and 50 per cent water. The volume added to each flask was 5 cc. unless otherwise stated. The different samples were allowed to incubate at 20°C. The

TABLE 1

*Bactericidal action of hexylresorcinol in milk**

CONCENTRATION OF HEXYLRESORCINOL	ORIGINAL	½ HOUR	1½ HOURS	3½ HOURS	7½ HOURS	24 HOURS
0	1,080,000	2,700,000	6,000,000	15,000,000	40,000,000	361,000,000
1:10,000		1,800,000	1,300,000	1,480,000	4,833,000	322,000,000
1:5,000		1,500,000	860,000	1,340,000	3,100,000	291,000,000
1:1,000		1,075,000	628,000	617,000	3,163,000	12,000,000
1:500		475,000	560,000	490,000	480,000	5,290,000
1:250		450,000	520,000	385,000	450,000	2,500,000
1:100		65,000	33,500	23,300	30,000	10,650

* Results expressed as number of bacteria per gram of food material.

TABLE 2

*Bactericidal action of hexylresorcinol in ground meat**

CONCENTRATION OF HEXYLRESORCINOL	ORIGINAL	48 HOURS	72 HOURS	144 HOURS
0	42,000	15,525,000,000	36,300,000,000	Spoiled
1:10,000		5,000,000,000	7,600,000,000	Spoiled
1:5,000		1,310,500,000	2,590,000,000	Spoiled
1:1,000		720,000,000	259,000,000	630,000,000
1:500		305,000,000	210,000,000	581,000,000
1:250		22,000,000	19,000,000	58,000,000
1:100		1,150,000	5,900	1,100

* Results expressed as number of bacteria per gram of food material.

number of bacteria was determined in the original food material, and like determinations were made, at stated intervals, both in the control and in the samples containing the preservative. All bacterial counts are expressed on the basis of the number of bacteria in 1 gram of the food material.

In table 1 are given the results of one of the experiments con-

ducted with milk. It will be noted that the hexylresorcinol in dilutions of 1:10,000 and 1:5,000 during twenty-four hours did not cause much reduction in the number of bacteria as compared with the control for the same length of time. Concentrations stronger than these greatly inhibited bacterial increase. The

TABLE 3

*Bactericidal action of hexylresorcinol in tomatoes**

CONCENTRATION OF HEXYLRESORCINOL	ORIGINAL	48 HOURS	96 HOURS	144 HOURS	264 HOURS
0	1,750,000†	15,700,000†	23,400,000†	30,000,000†	390,000,000†
1:10,000		2,355,000	30,800,000	34,500,000	22,900,000
1:5,000		310,000†	800,000	660,000	6,600
1:1,000		105,000†	110,000	16,000	3,100
1:500		45,000	17,000	10,000	600
1:250		38,500	4,000	1,100	300
1:100		7,000	1,200	200	Sterile

* Results expressed as number of bacteria per gram of food material.
† Mold.

TABLE 4

*Bactericidal action of hexylresorcinol in tomatoes**

CONCENTRATION OF HEXYLRESORCINOL	ORIGINAL	24 HOURS	144 HOURS	264 HOURS
0	18,848,000	3,000,000	27,000,000	309,000,000
1:10,000		600,000	26,000,000	31,000,000
1:5,000		500,000	240,000	370,000
1:1,000		50,000	Sterile	Sterile
1:500		10,000	Sterile	Sterile
1:250		4,000	Sterile	Sterile
1:100		2,000	Sterile	Sterile

* Results expressed as number of bacteria per gram of food material.

count at the end of twenty-four hours for the sample containing the hexylresorcinol in the dilution of 1:1,000 was 3.3 per cent of the control, and for the dilution of 1:100, 0.0028 per cent of the control.

Table 2 gives the results when ground lean beef was tested.

To 100 grams of finely ground meat, 10 cc. of the preservative were added and intimately mixed. None of the dilutions except 1:100 showed any reduction in the number of bacteria during one hundred forty-four hours.

Tables 3 and 4 give the results obtained for two samples of tomatoes. The tomatoes were chopped in very small pieces and the hexylresorcinol added. The samples used for the results reported in table 3 were very soft and moldy. Concentrations greater than 1:10,000 showed considerable reduction in the bacterial content. In table 4, it will be noted that when concentrations stronger than 1:5,000 were used, the tomatoes were sterile in one hundred forty-four hours and remained sterile for several

TABLE 5

*Bactericidal action of hexylresorcinol in eggs**

CONCENTRATION OF HEXYLRESORCINOL	ORIGINAL	24 HOURS	48 HOURS	144 HOURS
0	1,500,000	780,000,000	207,000,000	1,070,000,000
1:10,000		234,000,000	286,000,000	1,170,000,000
1:5,000		233,000,000	268,000,000	1,620,000,000
1:1,000		130,000,000	140,000,000	1,520,000,000
1:500		120,000,000	62,000,000	518,000,000
1:250		30,000,000	30,300,000	233,000,000
1:100		1,360,000	2,680,000	7,770,000

* Results expressed as number of bacteria per gram of food material.

weeks, after which time the samples were discarded. The tomatoes used in this set were quite ripe and soft, but contained no mold. The acidity of the tomatoes may account, in part, for the favorable results.

The next set of experiments was conducted on whole eggs. These were well beaten and then tested in the same manner as the other food products. The results for one of the samples are given in table 5, and indicate that the preservative was ineffective in the concentrations tried.

The results listed in tables 1 to 5 inclusive indicate that hexylresorcinol in dilutions of 1:250 or greater has a relatively poor preservative action for all of the foods tried except the tomatoes.

CONCLUSIONS

1. In protein foods, hexylresorcinol was not very effective in reducing the number of bacteria.

2. In an acid food like tomatoes, hexylresorcinol caused a marked reduction in the bacterial content.

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FURTHER STUDIES ON THE DIFFERENTIATION OF HUMAN AND ANIMAL STRAINS OF HEMOLYTIC STREPTOCOCCI[1]

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Received for publication September 2, 1932

The differentiation of hemolytic streptococci of human and animal origin is obviously a subject of great practical importance in the field of public health and hygiene. This problem has occupied the time of a number of workers and has been the subject of many publications. Since hemolytic streptococci have been found so generally in milk and milk products, most of these investigations were efforts to distinguish between human and bovine strains. While our knowledge concerning bovine strains of hemolytic streptococci has advanced rapidly, as a result, study of the streptococci from other species of animals has been neglected. No method has as yet been discovered by which it is possible to effect a final separation of human and animal strains.

Ayers (1916), Ayers, Johnson and Davis (1918), and Avery and Cullen (1919) demonstrated that many hemolytic streptococci of bovine origin could be distinguished from human strains by the final hydrogen-ion concentration produced in glucose broth. Ayers and Rupp (1922) found that the members of this group of bovine streptococci were able to hydrolyze sodium hippurate, while human strains did not attack this substance. Avery (1929) further showed that the high-acid-producing strains of bovine origin could be distinguished from human strains by the reduction of methylene blue by the former. Brown (1920) studied a

[1] The investigation reported in this paper was made in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

series of hemolytic cultures from bovine sources and found that the majority could be distinguished from human strains by final acidity in glucose broth, action on blood plates, and hemolysis in a fluid medium. Jones (1920) in the study of hemolytic streptococci from milk observed two groups, one a high-acid-producing type identical with mastitis streptococci, the other a low-acid-producing type more or less similar to human strains. The low-acid-producing type consistently failed to ferment salicin.

Brown, Frost and Shaw (1926) studied a large number of cultures isolated from certified milk, septic sore throat, and various pathological conditions in human beings. These cultures were examined for capsule production, hemolysis in fluid media, pathogenicity for mice, hydrolysis of sodium hippurate, and fermentative characters. The authors concluded that it was possible to distinguish human strains from bovine strains by these tests. The cultures were divided into nine groups on the basis of their reactions. One group was hemolytic in a fluid medium, failed to hydrolyze sodium hippurate, was pathogenic for mice, did not produce an acidity exceeding pH 4.8 in glucose broth, and generally produced acid from lactose, sucrose and salicin, but not from mannitol. These cultures were considered to be of human origin. This group was composed of cultures from infections in man, from cows thought to be responsible for epidemics of septic sore throat, and in a few instances from cases of mastitis in cows that had no connection with human disease. None of these cultures were from certified milk. The strains from certified milk all differed in one or more particulars from the human strains, and were considered to be of bovine origin.

The papers cited above indicate that bovine and human strains can in most instances be distinguished from each other with a fair degree of accuracy. However, certain strains of hemolytic streptococci are found in cows and other species of animals that are indistinguishable from human cultures by the criteria that have been used. Seeleman and Hadenfeldt (1930) studied certain strains of hemolytic streptococci from cases of mastitis and came to the conclusion that *Str. pyogenes* from cows is probably identical with *Str. pyogenes* of human origin. Hergesell (1931), in

studying the cultures described by Seeleman and Hadenfeldt, also came to the conclusion that they were identical with streptococci of human origin. Smith (1929) studied cultures from cows, horses and guinea pigs. These were largely low-acid-producing strains which failed to hydrolyze sodium hippurate. They were markedly hemolytic in fluid media and were pathogenic for guinea pigs. A detailed study of the cultural, biochemical, and serological characters of these strains failed to reveal any method whereby they could be distinguished from human cultures.

In an earlier paper (Edwards, 1930) attention was called to the fact that there was a large group of animal strains which could not be distinguished from human cultures by the methods that had been used previously. These strains were found to be present in cows and in other species of domestic animals. They produced a low acidity in glucose broth, failed to hydrolyze sodium hippurate, and were actively hemolytic in fluid media, thus possessing the characters ordinarily attributed to human strains. A large majority of these cultures could be differentiated from human streptococci by their action on sorbitol and trehalose. The human strains produced acid from trehalose, but failed to ferment sorbitol. The majority of the animal strains fermented sorbitol, but did not attack trehalose. In addition the animal strains as a rule produced a slightly higher acidity in glucose broth than did the human strains. The present paper is a continuation of this study, in which a larger number of strains is included and in which other morphological and biochemical characters are considered.

MATERIAL AND METHODS

The material to be reported here is based upon a study of 173 strains of animal, and 75 of human, origin. The sources of the strains are given in table 1. They were all actively hemolytic, low-acid-producing streptococci which failed to hydrolyze sodium hippurate. They all belonged to the group which has long been designated as *Str. pyogenes*.

The following characters are reported in the present paper: Acid-production from glucose, reduction of methylene blue, pres-

ence of capsules, and fermentative reactions. While a large number of fermentable substances have been tested, only those which are usually employed in the study of streptococci, and others that are of value in differentiation, are included.

TABLE 1

Source of cultures

Animal strains:	
Equine, os uteri	100
Equine, aborted fetuses	6
Equine, septicemia and arthritis	12
Equine, rhinitis	11
Equine, pneumonia	3
Equine, wound infection	1
Equine, *Str. equi* from Strangles	8
Bovine, septicemia	3
Bovine, placenta	1
Bovine, milk	2
Swine, septicemia	2
Swine, abortion	5
Swine, arthritis	1
Chicken, femur	11
Fox, pneumonia	2
Guinea pig, adenitis	4
Rabbit, septicemia	1
Human strains:	
Throat cultures, sore throat and scarlet fever suspects	22
Scarlet fever, stock cultures	22
Broncho pneumonia	9
Erysipelas	9
Mastoiditis	6
Puerperal fever	3
Meningitis	1
Peritonitis	1
Pleuritis	1
Smallpox	1

The methods used in testing for hemolysis, hydrolysis of sodium hippurate, final acidity in glucose broth, and fermentation of sugars have been previously described (Edwards, 1930). The presence of capsules was determined in moist india ink preparations of cultures grown in meat-infusion broth containing 20 per cent ascitic fluid.

In testing for the reduction of methylene blue the following medium was used:

Beef infusion	950 cc.
Casein digest	50 cc.
Bacto peptone	10 grams

The reaction was adjusted to pH 8.0 before sterilization. After eighteen hours incubation at 37.5°C., 0.1 cc. amounts of the cultures were used to inoculate tubes of the same broth to which sufficient sterile aqueous solution of methylene blue had been

TABLE 2

	NUMBER OF CULTURES	pH IN GLUCOSE BROTH	HYDROLYSIS OF SODIUM HIPPURATE	HEMOLYSIS	REDUCTION OF METHYLENE BLUE	CAPSULES	SALICIN	MANNITOL	LACTOSE	SORBITOL	TREHALOSE
Human	75	5.8	−	+	72− 3+	65− 10+	+	70− 5+	71+ 4−	−	+
Animal:											
Type A	159	5.2	−	+	−	+	+	−	+	+	−
Type B1	5	5.0	−	+	+	−	+	−	−	−	+
Type B2	1	5.0	−	+	+	−	+	−	+	−	+
Str. equi	8	5.0	−	+	−	+	+	−	−	−	−

added to give a final dye concentration of 0.000025 molar. The inoculated tubes containing the methylene blue were incubated at 37.5°C. for seventy-two hours, and observed at intervals of twenty-four hours for evidence of reduction. Cultures were recorded as positive which caused a complete decolorization of the medium except for a faint ring of blue at the surface of the broth.

RESULTS

The results of the tests are given in table 2. The animal streptococci were divided into four groups, on the basis of their reactions. The designations given these types by Ogura (1929)

in his work on equine streptococci were retained, since the same groups were observed among streptococci from various species of animals that Ogura found in horses. The first and largest group (type A) was composed of the strains which fermented sorbitol and failed to produce acid from trehalose. Type B was made up of cultures which, like the human strains, fermented trehalose but did not attack sorbitol. Type B was further divided into two types, B1, which did not produce acid from lactose, and B2, which fermented this sugar. The fourth type or group is designated as *Streptococcus equi*.

There was a difference in the final hydrogen ion concentration produced by the human and animal strains in glucose broth. The figures given in the table record the averages for the four different types. While there was a distinct difference in the acidity produced by the human and animal strains, this character could not be relied upon for differentiation, since there was considerable overlapping of the individual strains of different groups.

Str. equi attacked neither lactose, sorbitol nor trehalose. The number of strains (8) studied here is small. Holth (quoted by Jensen, 1911), Adsersen (1915), and Ogura (1929), together, examined more than 250 cultures of *Str. equi* and found them absolutely constant in their biochemical characters. Of the animal strains other than *Str. equi* studied here, 159, or 96 per cent (type A), fermented sorbitol, but did not attack trehalose. All of the human strains, on the contrary, produced acid from trehalose, but failed to ferment sorbitol. Six of the animal strains (types B1 and B2) gave the same reactions in sorbitol and trehalose as the human. One of the six produced acid from lactose, while the remaining 5 strains failed to attack this substance.

Since the Type B (1 and 2) animal strains possessed the same fermentative characters as the human, other means were sought to separate them. The method that gave the most promise was the methylene blue reduction test. All of the Type B animal strains reduced this dye under the conditions of the experiments, while only 3, or 4 per cent, of the human strains acted upon it. None of the sorbitol-fermenting Type A cultures reduced the dye, nor did *Str. equi* affect it.

Among the animal strains, Type A and *Str. equi* regularly produced capsules, while Types B1 and B2 did not. Certain of the Type B strains were subjected to serial passage through animals, but even after several passages there was no evidence of capsule formation. Capsules were observed on a few of the human cultures. No special effort was made to induce capsule formation in this group. While capsule production served to distinguish the Type A animal strains and *Str. equi* from the Type B animal strains, it did not serve to set them apart sharply from those which were of human origin.

DISCUSSION

Of the 173 animal strains, 96 per cent could be distinguished from the human strains by their fermentative reactions. The cultures of the group of animal streptococci designated here as Type A were absolutely constant in the characters studied. While the human strains varied somewhat in their reactions, particularly in the fermentation of lactose and mannitol, the sorbitol-fermenting cultures of Type A were quite consistent in these respects. No Type A cultures were found which fermented mannitol, and all of them produced acid from lactose.

The action of the low-acid-producing, hemolytic streptococci on sorbitol and trehalose was extremely interesting. A total of 315 cultures of this group was examined. With the exception of *Str. equi*, which fermented neither of these substances, the cultures all acted upon one or the other, but no strain fermented both.

The failure of *Str. equi* to ferment either sorbitol or trehalose, as well as its lack of ability to produce acid from lactose, established it as a definite bacteriological entity. Certain non-lactose-fermenting strains found in human infections have been designated by various writers as *Str. equi*. The problem of the differentiation of this species has been complicated further by the failure of various workers to recognize other streptococci, which are associated with *Str. equi*, in the lesions of strangles. This has caused some confusion; however, when the characters re-

ported here are used in differentiations, *Str. equi* stands out as a definite species, and should be so regarded.

While it was not possible to separate absolutely the Type B animal strains from those of human origin, the methylene blue reduction test very nearly served to differentiate them completely. All of the animal strains reduced the dye under the conditions of the experiments, while only 3 human strains gave this reaction. These tests were repeated several times and the results found to be constant. The dye reduction test was, of course, a quantitative one, a 0.000025 molar concentration of the dye being used in the differentiation. When as much as 0.00001 molar solution was employed all of the cultures caused visible reduction. When, on the other hand, a concentration of 0.00005 molar was used none of the strains reduced it completely. The medium used in these tests favored a luxuriant growth of the various organisms.

While the question of identity or exact relationship of the human to the Type B (1 and 2) strains may still remain in some doubt, it is quite probable that they are distinct and that the Type B strains are not in reality strains of human streptococci that have been transferred to animals. The Type B strains in general were of low virulence, and were found sometimes in tissues and organs that showed no pathologic changes. They were found associated with organisms of Type A or *Str. equi* in pathologic conditions in horses. It is probable that these cultures are usually saprophytic.

Another point of importance was the constant presence of capsules on the Type A animal strains. These capsules were large, prominent and easily demonstrated. As has already been stated, this group of animal strains is indistinguishable from human streptococci by the methods in general use. The Type A strains were indistinguishable from *Str. epidemicus* as characterized by Brown, Frost and Shaw (1926) and Pilot, Hallman and Davis (1930). The Type A animal strains not only formed capsules, but under appropriate conditions of culture formed large, spreading, mucoid colonies. This character usually has been considered peculiar to *Str. epidemicus*. While it was not intended to enter into a detailed discussion of the characters of

Str. epidemicus at this time, the writer is of the opinion that these animal strains are not the organism of septic sore throat. However, since we have received a number of cultures labelled *Str. epidemicus*, which were identical with the Type A animal strains, it is evident that they are often mistaken for this organism.

Opinions concerning capsule formation by the hemolytic streptococci may be greatly changed in the future. Brown and Kindwall (1928) demonstrated that cultures of non-encapsulated streptococci acquire capsules under appropriate treatment. Tunnicliff (1931) observed that the smooth forms of scarlet fever and erysipelas streptococci are encapsulated. Capsule production, or ability to form capsules under proper conditions, may be a much more common property of the hemolytic streptococci than has been generally supposed.

The question of infection of man with animal streptococci and the infection of animals with human streptococci is of great importance. The writer's opinion, based on the examination of more than 300 strains, is that the sorbitol-fermenting streptococci, which compose 95 per cent of the animal strains studied here, rarely, if ever, infect man. However, it has been demonstrated that human streptococci may at times infect animals. Jones and Little (1928) have demonstrated that cows may be infected with scarlet fever streptococci and transmit the disease to consumers of the milk. Furthermore, *Str. epidemicus*, which has long been considered as being of human origin, is known to infect cows and cause a severe mastitis (Brown and Orcutt, 1920). We have examined several strains from cows that were thought to be responsible for human epidemics and have found them to be of the human type. Infection of animals with human streptococci presents a situation which is the reverse of the condition prevailing in the Salmonella group where animal paratyphoids frequently infect man, while animals are rarely, if ever, infected with the human types.

SUMMARY AND CONCLUSIONS

In the study of 248 strains of hemolytic streptococci of human and animal origin, it was demonstrated that a large majority of

the strains that were of animal origin, namely 96 per cent, could be distinguished from the human cultures by their action on sorbitol and trehalose. The animal strains fermented sorbitol, but did not produce acid from trehalose. The human strains, on the contrary, fermented trehalose, but did not attack sorbitol. The small group of animal strains which resembled the human cultures in fermentative reactions all reduced methylene blue under suitable conditions. Under the same conditions only 4 per cent of the human strains reduced the dye. *Str. equi* was differentiated from all other human and animal strains by its inability to ferment lactose, sorbitol, or trehalose.

The sorbitol-fermenting animal strains (Type A) and *Str. equi* regularly produced capsules. The sorbitol-fermenting strains possessed the characters usually attributed to *Str. epidemicus*.

The sorbitol-fermenting strains of animal origin rarely, if ever, infect man. However, human streptococci are occasionally found in mastitis of cows.

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A LARGE-CELLED DISSOCIATIVE FORM OF ESCHERICHIA COLI AND ITS RELATION TO THE SO-CALLED PETTENKOFER BODIES

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Received for publication September 11, 1932

The abnormal cell types frequently found in cultures of bacteria have usually been considered as degeneration forms of the organism or as contaminating organisms. The idea that these abnormal forms are part of the life cycle of the organism has, however, been advanced by a number of bacteriologists. Löhnis (1921) has collected and reviewed the literature dealing with this phase of the subject.

Kuhn and Sternberg (1931) have reviewed the papers presented by Kuhn and others dealing with the occurrence of abnormal cell types in pure cultures of bacteria. Instead of classifying the variants according to the types of colonies produced or the antigenic properties, Kuhn classified them according to cell morphology. He has divided the cells into four types, A, B, C, and D. They are characterized as follows:

The B forms are the normally-occurring cell types. This type corresponds to the smooth type of other investigators and the cells are rods, cocci, or spirilla according to the culture that is being considered.

The C form is represented by the small, granular elements found in cultures of bacteria. This form is capable of passing through porcelain filters and can be cultivated for long periods in pure culture. Kuhn considers the C forms to be identical with the granules often observed in bacterial cells and which have been referred to as Much granules, Babes-Ernst granules, polar granules, or metachromatic granules. This type is perhaps similar to the G forms of Hadley (1931).

The long, filamentous, branched cells often seen in cultures of bacteria, Kuhn has called D or F forms.

The A form of Kuhn is reported as consisting of large, globular or coccoid cells which are produced most abundantly when the culture is treated in some special manner, but which may be seen in any culture of bacteria. By making conditions either more favorable for their development or less favorable for the development of the normal forms, the A forms may be secured in large number. Kuhn compares these forms with protozoa or myxomycetes and considers that they form a part of the life cycle of the bacteria. The A forms, according to Kuhn, pass through a series of changes, one of which is a cyst stage from which spores are liberated. These spores invade the normal cells, causing them to swell and burst, thus producing the phenomenon of lysis or bacteriophage action.

Koch (1927) investigated the occurrence of Kuhn's A forms and his results agreed with those secured by Kuhn. In addition Koch also agreed with the findings of Löhnis and with those of Enderlein. Kuhn stated that his data did not support the findings of these two investigators.

Mellon (1925a) studied the abnormal cells occurring in a culture of *Escherichia coli* secured from the urine in a case of pyelitis. The patient was receiving utropin and sodium acid phosphate, and long, filamentous cells as well as branched cells and many large coccoid cells were present in the urine. When the utropin and sodium acid phosphate were not administered, the morphology of the cells in the urine was identical with normal *Escherichia coli*. Mellon considered the large coccoid cells to be zygospores.

Mellon later (1925b) made additional observations on the coccoid forms of *Escherichia coli*. He found that these forms were produced when a normal culture of *Escherichia coli* was grown in veal infusion broth containing 1 per cent disodium glycerophosphate and 0.5 per cent sodium chloride. When the large cells were transferred to an agar slope only the coccoid forms developed. The author considered that the coccoid forms were the result of the reproduction of other coccoid forms whose antecedents were zygospores, and that long residence in broth had

stabilized them so that they reproduced as such under suitable conditions.

Hadley, Delves and Klimek (1931) noted that bodies resembling the A forms of Kuhn appeared in bacterial cultures undergoing dissociation. These workers were unable to observe the liberation of granules from the cells.

Nungester and Anderson (1931) studied an organism resembling *Escherichia coli*. The organism was secured from a case of empyema of the gall bladder. Smears from the specimen showed long, filamentous, branching forms which yielded bacillary forms of irregular sizes and shapes, as well as coccoid forms of various sizes, when cultured on laboratory media. With continued cultivation on laboratory media the pleomorphism disappeared and the morphology corresponded to that typical of *Escherichia coli*.

EXPERIMENTAL

Source of aberrant cells

In the investigation herein reported, the aberrant cells were first found on litmus agar plates smeared from a culture of *Escherichia coli* which had been carried in lithium chloride broth for twelve daily transfers. The plates smeared from each of the transfers, up to and including the twelfth, all showed abundant growth after twenty-four hours' incubation at 37°C. The appearance of the growth was normal except for a slightly rough appearance of the colonies.

Plates smeared from the thirteenth transfer failed to show any colonies after the twenty-four-hour incubation period, although broth tubes inoculated from the same material as the plates showed normal growth, as evidenced by turbidity. Likewise, the plates smeared from transfers 14 and 15 also failed to show growth. In each case the corresponding broth cultures had a heavy turbidity.

The three sets of plates which had no colonies after twenty-four hours were incubated for an additional twenty-four-hour period, and following this there were colonies present on all of the plates. The colonies were unlike the normal *Escherichia coli*

colonies, being round, smooth-edged, dome-shaped, opaque, and ranged from 2 to 5 mm. in diameter. In addition to the colonies the area over which the inoculum had been spread had a dull unpolished appearance as compared to the glistening appearance of the uninoculated agar.

The stained preparations made from the colonies described above revealed only large, round, coccus-like cells which were quite different from the cells of the original culture. These globular cells varied from 2 to 8 microns in diameter. Many of the cells were bean-shaped, and this was later found to be the result of a partial cell division. The globular cells occurred singly, in pairs, and in short chains. The cells were Gram-negative.

Characteristics of the aberrant culture

Examination of the lithium chloride broth from which the original plates were smeared revealed that the cells present were all rod-shaped and looked very much like the cells of the original culture of *Escherichia coli*. When these rod-shaped cells were planted on a lithium-chloride-free medium, the flora of the new culture was made up entirely of globular cells. A tube of lithium chloride broth inoculated at the same time had a flora made up entirely of rod-shaped cells. The rod-shaped cells were motile and the globular cells were non-motile. Attempts to demonstrate flagella by staining methods were always successful with the rod-shaped cells and never successful with the globular cells. The loss and acquisition of flagella occurred regularly with the change in morphology and was present on both solid and liquid media. The factor which controlled the morphology was apparently lithium chloride.

The possible effects of the medium on the morphology of the cells may be explained on either of the following assumptions: (1) that the original culture contained more than one species of organism and that the media used had a selective action for one of the types present; (2) that the transformation of one cell type to the other was a function of the composition of the medium; or (3) that the globular cells represented myxomycete-like or

protozoa-like stages in a life cycle as suggested by Kuhn. Experiments were set up to study these different phases of the problem.

Living preparations of the organism were made, either by inoculating a small droplet of broth and incubating it over a moist chamber, or by placing a small drop of nutrient agar on the cover slip and smearing the inoculum over this after the agar had solidified. Lithium chloride was included in the media in a concentration of 0.35 per cent. The inoculated preparations were under continuous examination with the oil-immersion objective, and the development of the individual cells could be easily followed.

Effect of media on morphology

The cells used for inoculum had been grown for a number of transfers in lithium chloride broth, and were all rod-shaped. Inoculations were made in lithium chloride broth and agar, and the changes in morphology were followed. In every instance the rod-shaped cells were seen to reproduce as rods. The original culture was motile and the cells in the new cultures were also motile.

Rod-shaped cells from the same source as that used in the above observations were now inoculated into broth and agar that were free from lithium chloride. Soon after inoculation the individual cells could be seen to begin to swell and continue to swell until they had all become globular-shaped. Accompanying this swelling was the gradual loss of motility, and ordinarily no motile cells could be found among the globular cells. However, an occasional motile globular cell was seen in the cultures. The newly formed globular cells could be observed to multiply by division and the daughter cells were also globular-shaped. Dividing cells frequently possessed an amoeboid motion, but this was wholly different from motility as it is ordinarily considered among bacteria.

Preparations were now inoculated with globular cells which had been cultivated in lithium chloride free media. The first media inoculated were free of lithium chloride, and the cells

developing in it were always non-motile, globular-shaped cells. When the globular cells were inoculated into media containing lithium chloride, the following changes were noted: The globular cells could be seen to shrink in size and cell division was soon evident. With each cell division the cells became more rod-shaped. After three or four divisions had occurred, the cells were all rod-shaped and motile.

All of the experiments were now repeated using a culture of the organism (from lithium chloride broth) which had been purified by single cell isolation. In every instance the results secured were exactly the same as those secured when using cultures purified by plating methods.

The effect of the osmotic pressure of the medium on the development of the globular cells was next studied. A series of broth cultures containing various materials in concentrations isotonic to the lithium chloride broth originally used were inoculated. The materials included in the series were lithium chloride, sodium chloride, potassium chloride, and sucrose. Plain broth was also used in the series. The materials to be added were sterilized in 10 per cent aqueous solution and added to the sterile broth. The culture used for inoculum was purified by single cell isolation from a lithium chloride broth culture.

Rod-shaped cells developed in the tubes of broth containing lithium chloride and sodium chloride, while globular cells were present in the tubes containing potassium chloride and sucrose, and in the tube of plain broth. This condition existed for many transfers with little or no change in the morphology of the cells. These results indicate that the osmotic pressure of the medium does not influence the development of the abnormal cells, and that there is some other factor controlling this change.

The aberrant culture had the same biochemical properties as the typical culture in glucose, lactose, and sucrose broths. It also gave the typical *Es. coli* appearance on Endo agar, though the colonies were smaller and developed more slowly.

Reversion of the aberrant form to normal type

A culture of the aberrant cells was carried in 1 per cent lactose broth for a number of transfers. The cells at first were globular-

shaped, but, as the transfers continued, there was a definite tendency for the cells to be more and more rod-shaped. After nine daily transfers the cells present were similar to the cells of normal *Escherichia coli*. When they were transferred to various other media there was no change in the morphology of the cells developing. The ability of the rod-shaped cells to change to globular cells had apparently been lost. This reversion was also accomplished by serial transfers in plain nutrient broth.

Forms noted by other workers

Such phenomena as cyst formation with the liberation of "spores," the fusion of cells, and the formation of minute forms (the C forms of Kuhn) were not observed in this work. A number of filtration experiments were conducted with cultures of the large-celled form with negative results.

SUMMARY

The data presented describe an aberrant cell type, which is probably identical with the Pettenkofer bodies of Kuhn, secured from a culture of *Escherichia coli* which had been carried in lithium chloride broth for thirteen days with daily transfers, and then streaked on agar. The aberrant cells were globules and were found to change directly to rods when grown on a medium containing lithium chloride, and to change again to the globular form when grown on lithium chloride free medium. Accompanying this change in morphology was the loss of motility of the globular cells.

Cultivation of the aberrant types in various broths made isotonic to the original lithium chloride broth yielded results which indicated that osmotic pressure was not the controlling factor in the occurrence of these cells.

The aberrant types reverted permanently to normal *Escherichia coli* when grown in plain broth with serial transfers for a period of time, and when grown in lactose broth through nine daily transfers.

The biochemical properties of the aberrant types were identical with those of the original *Escherichia coli* in glucose, lactose, and

sucrose broths and on Endo agar. Colony production on agar media was much slower with the aberrant type culture; the colonies were much smaller and different in appearance.

All of the experiments were repeated, using cultures purified by single cell isolation, and the same results were obtained.

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SYMPOSIUM ON BACTERIAL VARIATION

Abstracts of Certain Papers Presented before the Society of American Bacteriologists at Ann Arbor, December 28, 1932

OBSERVATIONS ON AMORPHOUS PHASES OF BACTERIA[1]

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It seems best to limit our contribution to this series of discussions to a description of the results we have been able to obtain in our laboratory, describing, as briefly as is possible without implying sweeping or erroneous generalizations, (1) the character of the changes observed in amorphous phases of the life cycle, (2) their varying origins, and (3) the forms resulting from such formless matter, so far as we have been able to determine them.

Readers may question the repeated use of the descriptive adjectives, "amorphous" and "formless" and the avoidance of the designation symplasm. It is probably wise, however, not to complicate the conclusions and generalizations of life cycle studies relative to filterability and invisible phases of bacteria by extending the term symplasm to include the huge and often bizarre masses (figs. 2 and 3) and the compact capsular bodies which are internally amorphous but distinctly visible and which may coexist with the more diffuse and invisible phases now generally implied in the term symplasm.

[1] Grants from the New York Academy of Sciences, the Society of Sigma Xi and the Joseph Henry Fund of the Washington Academy of Sciences have made possible the purchase of single cell micro-manipulator and motion picture apparatus for this investigation and secured the coöperative assistance of Jane Carpenter, Russell Sage College, and Theodore Hausmann, Concordia College in cultural and photographic work.

The observations upon which this report on amorphous forms of bacteria are based were made on hanging drop studies of an aerobic, non-pigmented spore-forming rod (fig. 1) isolated in March, 1932, from floor wax; and they are supported by many similar manifestations in two pigmented, rod-shaped bacteria (one a maroon-colored, non-spore organism and the other a pink spore-former) which have been under cultivation in our laboratory for two and four years, respectively. The pigmented organisms are as yet unidentified; the non-pigmented organism was described at the summer meeting of the Society of American Bacteriologists at Syracuse, in June, 1932, under the name *Bacillus polygenesis*, a name suggesting the surprisingly varied ways in which new cells are formed by this organism, most of which cannot be included in this report, dealing only with the amorphous phases we have observed.

Had the investigations of these phases, involving the formation of seemingly non-cellular masses, been described at the end of the first six months of intensive hanging drop observations of *Bacillus polygenesis*, we would have been tempted to claim a much more definite or obligatory sequence of growth phases. Further study, especially repeated observations of the same individual cells or growth areas for prolonged periods—in many cases throughout ten to twenty weeks—has made it evident that morphologically indistinguishable bodies may be formed in several different ways, and despite their differing origins, *may* develop along similar lines. For example, to use a phase of *Bacillus polygenesis* not included in this report, "balloon bodies" may arise in an apparently ordinary chain of vegetative cells by fission, they may develop from greatly enlarged free spindle cells, or they may arise by the looping around of a long rod and the absorpton of a contiguous cell at the attached unlooped end; and, all of these types of "balloon bodies" may look exactly alike and all may produce two to four nuclei or bodies which fill the "balloon" cell, as spores do a mold sporangium.

Or, to illustrate from the amorphous phases pictured in this paper, tiny globoid cells (fig. 4a) identical in shape, size, and type and degree of motility may develop from such various phases as (1)

diffuse amorphous matter (fig. 5), (2) dense capsular bodies (figs. 6 and 7), (3) from definite but readily disintegrated masses (fig. 8), or (4) as gonidia from what is apparently an ordinary vegetative rod; and these apparently similar globoid cells *may* all produce like results or stages, such as the disk-like bodies of amorphous matter (fig. 10).

It is commonly recognized that the amorphous phases may be missed entirely in stained preparations; there, even the least tenuous phases are not likely to be remarked by investigators, so sensitive are we to the charge of being misled by artifacts. Most of the amorphous material is dissolved or shattered by the staining processes ordinarily used. Even when we omit fixation by heat and use weak stains (1 per cent neutral red or water solutions of methylene blue) there is little to be seen, other than the ordinary formed cells, which would not ordinarily be classed as débris, "extraneous matter" or artifacts.

With the hanging drop preparations we find a wholly different condition, for there we can see the changes take place instead of deducing them from stained slides. And a return to the study of individual living cells makes one realize that bacteriology has been handicapped by the common dependence on what has seemed our indispensable mechanical aid, bacterial stains. There is, we find, much to be gained by the "Chinese patience" type of continued and more or less continuous observation used by some of our early investigators. Fortunately, the motion picture camera not only provides an assistant for such grilling work but confirms our observations by the photographic records.[2]

It might not be out of place to mention at this point the impressive effect obtained by using in hanging drop studies the microscope part of a motion picture set-up as an observation microscope—even though no photographic record is desired. The long tube of the observation eyepiece makes it possible to get higher magnification with the high dry objective than we can secure with an oil immersion objective and a X10 eyepiece, and amorphous

[2] The photographs herein included owe most of their value to Mr. Maurice Ricker, who can make something of even the poorest efforts of amateur photographers.

material, whether finely dispersed matter or tenuous slime, can be distinguished with the illumination used with the motion picture microscope. The existence of invisible phases of bacteria cannot be challenged by any one who, so aided, focuses on an apparently blank field in a hanging drop where no moving or formed cell is visible and has not been for weeks, and observes in that field the appearance of such amorphous matter and the emergence of numbers of formed cells.

CHARACTER AND OBSERVED CHANGES

In our cultures of *B. polygenesis* the multiplication of amorphous matter was first observed in a hanging drop preparation several days old which had been made from a twenty-four-hour glucose broth culture. The tube used was one of a set of eight tubes in our routine daily transfers of this organism. Two of the sister tubes had failed to grow and they were set aside and one of the tubes of usual turbid appearance was used for the hanging drop slides. In one of the slides, we found four or five days later, an indefinite mass of wholly amorphous material more than two fields in diameter, with vaguely irregular margins and varying in thickness or depth (fig. 5). The whole mass seemed somewhat vibrant (for want of a better term)—not as a mass, but as if a gentle simmering condition existed within it. Several observers confirmed this impression; unfortunately, our motion picture film could not record this slight motion, although evidence of activity is to be seen at the "craters" or peaks which spotted the mass, a definite pulsating or sometimes a rapid "winking" effect being observed at those points.

This amorphous mass spread or increased along its free margins, but observers received the impression that there was also a decided increase from below the visible surface of the mass, which accounted, in part, for the indefinite but widening marginal growth. Later, in studying the more opaque and firm-looking masses which developed in older hanging drops, it was found that the masses seemed to explode, as if from internal or underlying stimulus or force. This "explosion" sometimes increased greatly (2 to 3 diameters) the areas of a mass and this phenomenon

was sometimes accompanied by a period of rapid growth, of the same type which was carried on before the explosion occurred. The rate at which bacterial growth may be increased by this method of reproduction must be enormous and may account for the rapid increase in turbidity when no-growth tubes do begin to manifest growth. Figures 10 and 11 were taken less than twenty-four hours apart.

The brown or black masses of twisted, irregular rods (fig. 12), whether developing late as rods in such an amorphous mass (fig. 13), or whether developing throughout as rods only, exhibited this same explosive activity and figure 14 shows a field as it appeared after a mass like figure 12 had exploded.

These explosions occur also in some of the firmer or more solid-looking masses of amorphous material. Figure 15 pictures a smooth-looking body which showed a few indefinite lines and ridges on its surface and after continuing without change for over five weeks suddenly split over-night, presenting the next morning the shattered picture seen in the photograph. The broken pieces moved farther apart gradually, as shown by the changes in figure 16 photographed about two hours later.

In this explosion, eight or nine soft mobile particles, generally ellipsoidal in shape, but elongating later into slightly longer bodies as they became motile and much less distinct in outline than ordinary rods, were slowly freed from the center of the exploded mass. Two of these bodies appear as slight lumps or elevations in the central depression in figure 15 and one only remains in figure 16 two hours later. No motile organisms and no formed rods or other cells had been seen in this drop for over seven weeks. Yet, now, suddenly, moving bodies were present. They moved slowly; one explored all the ridges of the three lower lobes of the mass and then, still slowly, moved off into space, becoming suddenly quite active and darting here and there. It settled down once, as if wholly quiescent, but suddenly resumed active vibratory movement, and in about ten minutes became immotile about three fields from its origin, where it has now remained without change for four weeks (fig. 4a). In the meantime other pulsating nodules had scattered over the field and in the near neighborhood,

one of which may be seen in the lower right corner of figure 16. One of them divided into two cells (fig. 4b) within twenty-four hours.

Small fragments of the main mass also appeared scattered over the field, and these masses varied greatly in size, shape and texture as illustrated by the group in figure 18. As usual, in such cases, they varied from the minutest particle visible with the oil immersion lens to fragments half the diameter of the original mass and often half the diameter of the microscope field. Such fragments may be soft-looking bodies of rounded contours, but are often sharply angulated and many seem transparent, suggesting shattered glass. These bodies show evidences of growth, rounded nodules appearing at the corners, edges, and, sometimes along the entire margin. Occasionally, especially in the thinner, flatter masses, the nodules seem to push out from within the mass, and figure B shows a series of drawings copied from the film shown at the Ann Arbor meeting of the Society of American Bacteriologists. In the film it is possible to observe not only the formation and gradual increase in the number of these nodules or globoid bodies (from 1 to 5) and the coincident disintegration or disappearance of the mass in which they were formed, but to observe, also, the escape of two of the five bodies from their fixed sites, even though their line of escape was slightly out of focus. Unfortunately, these escaping globoids could not be seen in the photographs reproduced from any single exposure in the film record and so cannot be shown here.

The first of the two cells which escaped in figure 8 vibrated with evident pulling or tugging efforts to free itself for over twenty-four hours, swinging in varying arcs up to 180° for the last three hours of that period. After an interval of three hours the second of the escaping cells increased the vibratory efforts it had been making for some hours, and after a three-hour period of active effort also swam away. The total time period of the events here described—from the time when only one definite globoid body was observed, through the formation of four additional bodies and the loss of visibility in the foundation mass and the escape of two of the globoid bodies—extended over five days. This, as such observa-

tions go in hanging drops studies of these amorphous phases, is a rather rapid change.

OBSERVED ORIGINS OF AMORPHOUS PHASES

Several different immediate origins have been observed for these masses of amorphous material.

1. They may arise from single globoid bodies which surround themselves with what seems to be a gelatinous ring or envelope and then flatten out—sometimes in a few hours—into a soft-looking, apparently semi-fluid mass, with modelled contours and entire to more or less crenate margins resembling very strikingly gross colony appearances on agar. These masses, however, it must be emphasized, show no evidence of formed cells, though pulsating points may appear; these pulsating areas may shift, however, without releasing any free cells, and they may be related to the explosive condition previously described. These masses are often beautifully modelled and geometrically interesting (fig. 9). As they age, they may become rough-surfaced, even coarsely warty and scaly. These masses tend to explode and reproduce by tiny globoid bodies—which may repeat the same history.

Sometimes, however, the completed mass becomes gradually ridged or lined and a more or less complete dark brown or black mass results (fig. 13). These dark masses usually explode and follow the development described under (3) in the later section of this paper headed "Resulting Forms."

2. These amorphous masses may arise as secondary or later stages of the single coccoid cells, following the development of the initial globoid body into a preliminary twisted mass of dark cells, around which an encircling ring or layer of amorphous material forms (figs. 10 and 11). In this case the small globoid cells usually become dark in appearance (yellowish to tan-brown by green filter light) and divide into 2, 3 or even 5 or 6 cells, often in a few hours in hanging drops which are not more than five to seven days old. By the time more than 3 cells are distinctly visible the cells usually arrange themselves in a ring, circular or compressed, or into a skein-like twist. The cells soon show an unusual amount

of mobility but no motility. They lie twisted and crowded against each other (fig. A) and show constantly shifting bulbous contours and positions, writhing slowly around each other within the skein-like cluster of cells and never loosening or losing a cell from the mass. A further report of this phase is contemplated in the near future, but it must here be considered only as the core or nucleus around which a layer of amorphous matter develops, such layers extending for several times the diameter of the dark skein-like core, the spread being hastened by the explosions previously described. The core may be very irregular in outline, or beautifully symmetrical, as in figure A, a drawing made from the film view of the dark body in the lower right corner of figure 10. This twisted core may apparently continue to add more twisted rods until a huge mass develops (fig. 12), or it may act as a nucleus around which amorphous matter develops (fig. 10). This combination of an originally cellular core and an amorphous rim is much the commonest type of amorphous growth in *B. polygenesis.*

3. Small groups of the globoid bodies may combine when lying close together and form a soft-looking disk, differing mainly in the crenations of the margin and the bulbous modelling of the surface, these corresponding apparently to the number of globoid bodies originally present. These bodies often space themselves with remarkable symmetry, as many as twelve bodies sometimes forming an evenly crenate edge. While this method may not be really different from that in which a single globoid body begins the development of a mass of amorphous material it results finally in very different-looking masses, having rougher and more scaly surfaces.

4. The formation of amorphous matter by the fusion of formed cells, as in Myxobacterium, has not been observed. If no-growth tubes of *B. polygenesis* are kept until growth appears, we often find large irregular masses or patches of non-cellular matter. These may be crystalline and show curious fracture contours, or they may be very thin flat patches, often irregular in outline, as if torn from a film-layer or lining in the test tube. These flat patches are dead-looking, as if they represented degenerated material; but vibrating globoid bodies such as those in figure 4 de-

velop in them as well as in the crystalline fragments and in the more viscous or protoplasmic-looking bodies shown in figure 8. The number of globoid cells formed in such masses are relatively few, sometimes but one or two in patches as wide as the field of the microscope, and the whole effect is very unlike that of fused plasmodial masses in other microörganisms.

5. The formation of amorphous stages from vegetative cells has not been observed in *B. polygenesis*. In another organism, however, our maroon organism, we observed shorter forms which flattened out into concave disks, the cell material showing six or more distinct nodules, which soon became lost in the homogeneous character of the amorphous mass. These masses were always relatively small when compared with those obtained in *B. polygenesis*.

Occasionally some of the smaller disks (2 to 3 times the diameter of the original cell) reverted to the original short rod. This organism furnishes our only indication that ordinary rod-forms are directly related to amorphous phases. At any rate, it suggests a state of "flux" or instability not generally recognized, and such cell mobility and reversibility deserves further study.

RESULTING FORMS

1. The resulting or final form developing from these amorphous masses is characteristically a small globoid body (fig. 4a) which early shows a more transparent ring or covering. When formed from the indefinite masses in figure 5 they seem to develop without a motile period, dividing into two cells and making either skein-like twists or compact masses, often remarkably circular in outline, which in time rupture and show globoid bodies in the interior (fig. 15) or they reproduce indefinite masses similar to those from which they arose.

2. Definitely outlined masses of amorphous material, ranging from slender wand-like bodies to large irregular masses greater that the diameter of the field liberate motile globoid bodies, which may shortly settle down (twenty minutes to one to two hours in observed cases), dividing slowly into 2 or 3 cells or remaining inactive. They may develop into soft irregular masses similar to

the fragments which may come from the same mass and slowly become nodulated. The globoid bodies formed on or in these masses are usually scattered and few in number, and may be only 1 to 3 to a mass half the diameter of the oil immersion field. Figure 3 shows part of a mass which was three times the diameter of the field, which has crenations of the edge corresponding in general to the globoid bodies forming near the edge. Often the globoid bodies resemble tear-drop extrusions along the margin of the amorphous mass. Occasionally a marginal nodule narrows into a soft-elongated body, pulsating slightly for prolonged periods (one or more days), and may finally move away. Usually such pulsating bodies become quiescent and seem to be reunited to the mass, although their previous activity appears to be self initiated.

3. A third and common development of the amorphous bodies is the formation of dark lines—more or less concentric rings being formed inside the margin (fig. 13). These lines correspond to irregular rod-like bodies which show more or less activity within themselves and with each other, but have never been observed to leave the mass. Several dozen black masses, which have been observed for weeks at a time, have seemed to crack or explode, either gradually with open areas being formed as at one end of figure 12 or suddenly, almost as if the mass had been segmented, as a pie is cut into wedge-like sections. These sections may later explode still more and the whole compact mass of blackish material is then represented by somewhat paler rod-like units (fig. 14), very vague in outline, which gradually fade into almost colorless globoid bodies, with the usual transparent border. Some of these remain at the sites occupied by the rods from which they developed, but some of them elongate into sluggish rod-like bodies and move out of the field. They are, however, smaller and less definite than the usual vegetative rod and apparently form globoid bodies again, at least in our aged hanging drops. What they would do in different environmental conditions has not been ascertained.[3] In no case has the *original* type of rod been ob-

[3] The difficulties in carrying out continued observations of this kind may not be evident. We use the Leitz moist chambers so as to lessen the chances of losing the drop by drying or by cracking the coverglass. The number of drops studied

tained directly or indirectly from these or any other amorphous phases.

Securing such formless stages of organisms seems, from our experience relatively easy. Of course, we worked with three organisms apparently accustomed to ordinary room temperatures although some work by Itzkowitz in our laboratory indicates that such amorphous phases may be induced in at least one organism familiar to all bacteriologists by using high temperature. There is an advantage in working with an organism developing at room temperatures as currents are set up by heating the hanging drops, even when we use thermostat control heating devices. These currents cause shifts in the positions of the organism and induce movements which make motion picture photography impossible.

Occasional no-growth tubes, as mentioned, usually furnish good material for study. Aged normal cultures may be equally good sources, but even twenty-four-hour cultures may reveal occasional masses, mainly of the small angulated particles, especially at the time when other contemporary broth tubes fail to show growth. We have found the most numerous and most varied kinds of amorphous growth in old hanging drop cultures. As the motile forms disappear and all movement ceases, it is possible to chart the hanging drop field indirectly by mechanical stage readings and keep several dozen amorphous masses under observation.

After some days in the hanging drop, the growth changes are slow, and observers may fail to note slight changes in morphology and conclude that activity will not be resumed. The continuance of patient observation of such material is justified by such unexpected phenomena as the formation emergence of motile cells from rupturing formless material (fig. 15), a phase first observed after untold hours of patient watching.

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at a time is limited by the costs of these chambers ($4.00 each) and by the fact that these stages may be interminably slow, sometimes continuing weeks with little observable change. Since prolonged observation may yield growth weeks after all growth had apparently ceased one hesitates to discard any old slide lest significant phases be missed.

The foregoing descriptions of increasing masses of material, composed of particles or units too small to be visible as entities, and the formation of motile, growing bodies from such amorphous material must be viewed as giving support to the claims of the existence of invisible, living forms or stages of bacteria. This statement should be guarded, probably, by adding the warning that we must not expect too prompt evidences of activity—in the formless matter or in the bodies preceding or following such amorphous phases—for in hanging drop cultures, at least, activity may apparently be suspended for prolonged periods, not only of days but even months.

Attention might be called again to the possible high rates of bacterial multiplication in the amorphous stages described, dozens of formed cells suddenly appearing in fields up to that time devoid of all signs of life. The unsuspected presence of invisible antecedents of these visible organisms brings up the disturbing question of the reliability value of a single cell isolation. When organisms develop so numerously in an area which the microscope declares devoid of cells, what living units may attend unseen the single cell we select with such care with our micro-manipulator! Successful pure strain isolation must often depend as much upon the chance absence of such phases as upon the skill of the worker.

No attempt has been made to "fix" any of the amorphous phases described. So far they have been but one of several phases under observation, and the more active and rapidly changing stages have limited the attention given these forms.

The relation of these observations to filtrable stages of bacteria is obvious; but no filtration tests have been made, because of the lack of time as well as equipment necessary to do reliable filtration experiments. It would, from our experience, seem as if filterability might be more a question of when a culture was filtered, rather than whether the organism is or is not filtrable, and that obtaining such phases by filtration is more a matter of the amount of formless matter present, rather than a question of its presence or absence.

The fact that old preparations give us the most varied types of amorphous manifestations does not militate against the importance

of these phases in nature, for no one knows what "natural conditions" for such organisms as bacteria really are—nor what phases bacteria may show in the varied environmental conditions they meet.

EXPLANATION OF THE ILLUSTRATIONS

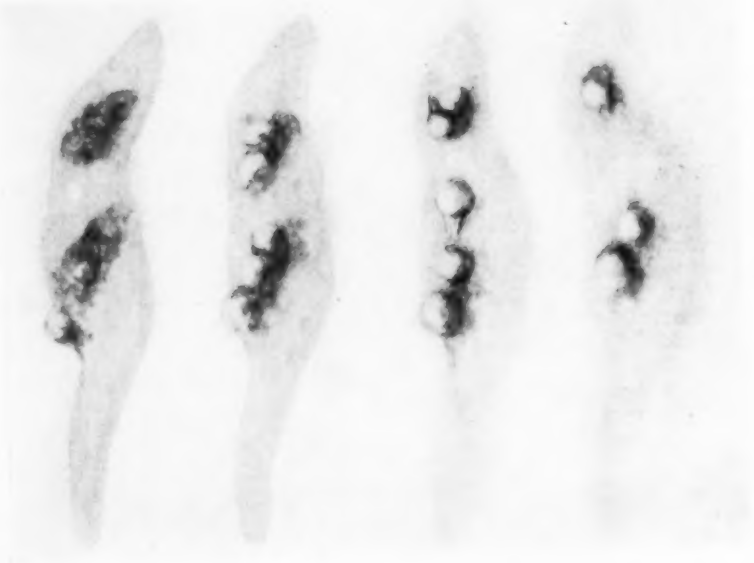
These photographs of *B. polygenesis* are enlargements from 16 mm. motion picture films and show phases obtained in Leitz moist chamber preparations of glucose broth. The vegetative rods (fig. 1) range from 3 to 8 microns; all the photographs have the same magnification as the rods in figure 1.

PLATE 1

FIG. A. Drawings showing the tightly twisted character of mobile cells which may form huge masses of such cells or may become the center of an amorphous mass (fig. 10). The snail-like mass at the left is an enlargement of the group of cells in the lower left corner of figure 10.

FIG. B. Four drawings of the development described for figure 8. In the third picture the artist has tried to indicate the visible pull of the escaping globoid bodies.

A



B

(Jean Broadhurst: Amorphous phases of bacteria.)

PLATE 2

FIG. 1. Vegetative rods in a five-day moist chamber preparation made from a twenty-four-hour glucose broth culture.

FIG. 2. An irregular, firm-surfaced mass of wholly amorphous matter developing in a ten-week moist chamber preparation.

FIG. 3. A softer, more plastic mass than figure 2, appearing more frequently than figure 2 in the same chamber.

FIG. 4A. A typical globoid body with the characteristic gelatinous ring or covering. This is the escaping globoid body described in the text in connection with figures 15 and 16.

FIG. 4B. One of the globoid bodies liberated shortly after 4a, and promptly showing division into 2 cells.

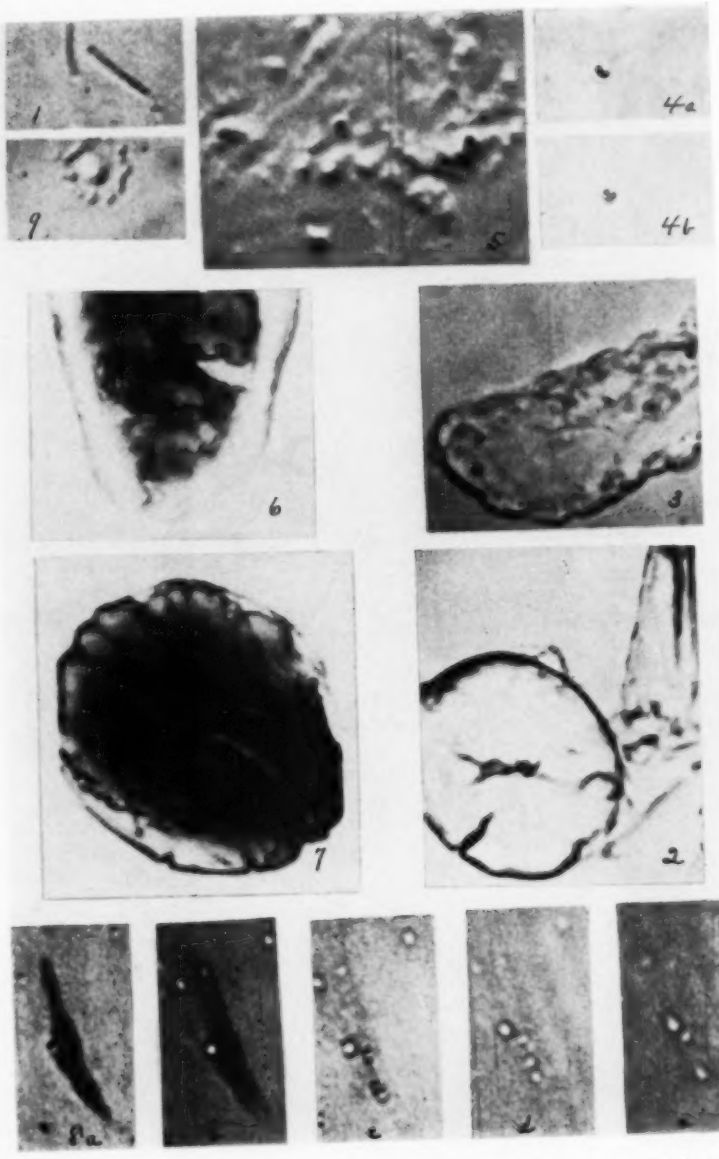
FIG. 5. Diffuse, spreading phase, resembling a floury paste, obtained during the fourth week after the isolation of *B. polygenesis* and not observed since.

FIG. 6. Part of a dense capsulated body with a rather uncommon pale peripheral area. Two soft bodies may be discerned in the split at the lower end.

FIG. 7. A more common type of capsulated body. These may be perfectly rounded and dark from the earliest stages; usually the early phases give the impression of a compact mass of more or less rounded units.

FIG. 8. Five photographs showing a spindle-shaped protoplasmic-looking mass which added 4 globoid bodies to the one present in *a*. Note the gradual disappearance of the protoplasmic mass and the loss of two of the globoid bodies in *c*, *d*, and *e*.

FIG. 9. A very common type of amorphous matter. As these continue to enlarge they may become very rough surfaced with several craters or explosive centers.



(Jean Broadhurst: Amorphous phases of bacteria.)

PLATE 3

FIG. 10. Amorphous mass developing around a center of dark twisted cells, which developed rapidly in an eight-day moist chamber. A twist lacking surrounding amorphous matter is shown in the lower right hand corner.

FIG. 11. Photograph taken about twenty hours after figure 10, during which interval an explosion spread the enveloping material beyond the limits of the microscope field.

FIG. 12. A mass of dark twisted cells, a type of growth common in moist chambers four or more weeks old. The open spaces at the upper end are due to partial explosions which have occurred.

FIG. 13. A wholly amorphous mass which later became lined by the development of dark, irregular rods; twelve-week preparation.

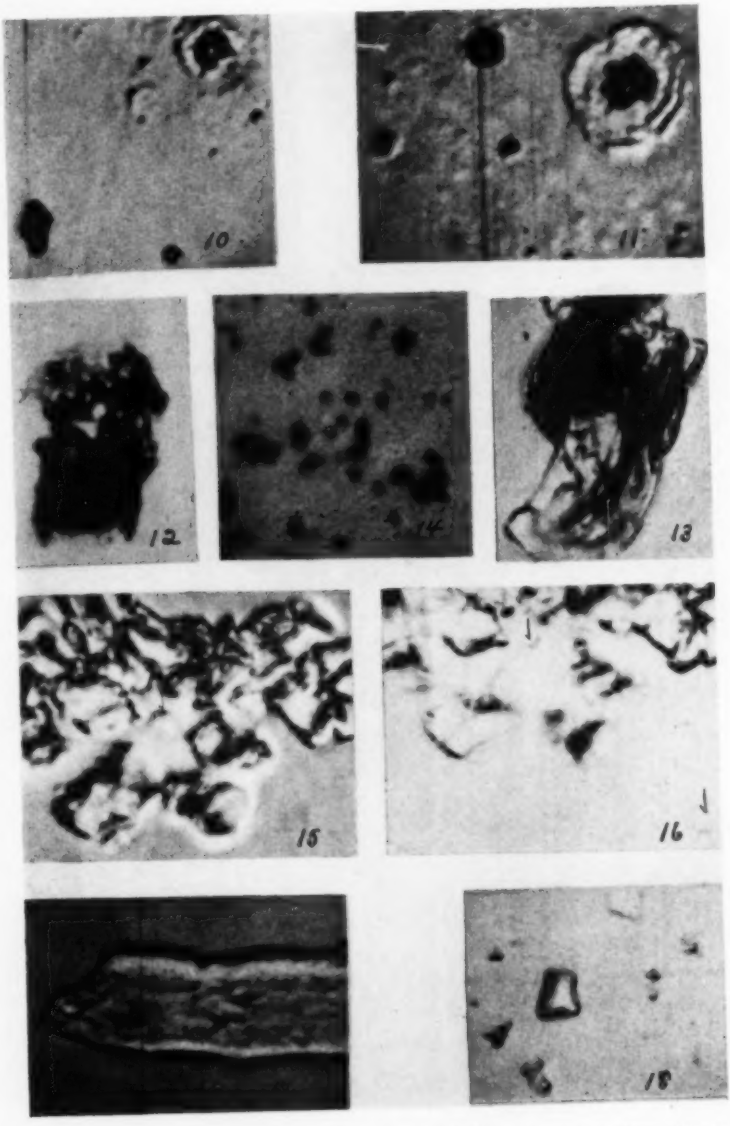
FIG. 14. A small area of a mass originally like figure 12 exploded into blurred rod-like bodies, which gradually fade (twenty-four to seventy-two hours) into pale globoid bodies.

FIG. 15. An amorphous mass which suddenly shattered into large segments. Two soft indefinite bodies which later became motile globoid bodies may be seen just below the arrow.

FIG. 16. Further separation of the split masses in figure 15 by propulsion from within. But one of the pre-globoid bodies seen in figure 15 is now left in the central area. One which freed itself elsewhere from the main mass may be discerned in the lower right corner.

FIG. 17. An amorphous mass photographed to emphasize the doughy or "solid" consistency of such masses, even when not in capsuled or sclerotic condition; a twelve-week preparation.

FIG. 18. A photograph taken six hours later than figure 16, showing a few of the varying types of small fragments, some of which are developing globoid bodies at the corners or edges, such fragments may be found 2 or 3 fields from the main mass.



(Jean Broadhurst: Amorphous phases of bacteria.)

II. SOME PITFALLS IN BACTERIOLOGY[1]

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Problems connected with the variability of bacteria have always confronted biologists but, within recent years, all phases of the question have been brought to our attention more insistently than heretofore from numerous sources. One finds it difficult to orient his pure culture studies in the light of modern research. Under such circumstances it becomes expedient to go back to the older concepts—the more firmly established positions—and strike out from these into the no-man's land of partly consolidated advances and thence into the more hotly contested fields. There one is left to decide for himself what may be regarded as conquered territory and what must still be wrung from the limbo of the unknown and what must be rejected as wholly untenable. There are no guide posts in no-man's land. Careful observation, knowledge of the pitfalls and traps, and sound judgement and, above all, the experimental method, must serve instead. It is the object of this paper to set up a few guide posts in no-man's land, an admittedly hazardous project, subjecting the pathfinder to shots from both sides.

Without intending to influence the interpretation placed by anyone upon the reports which come to his attention and without any criticism, implied or otherwise, of the work of any single investigator, it is felt desirable at this point to indicate some of the pitfalls and sources of error which closely beset every bacteriologist in his daily work. Then, one attempting to evaluate recent reports upon the subject will know at least where question marks may well be interpolated. It may also prove of assistance to the worker just entering the field. All of the sources of error men-

[1] From the Bacteriological Laboratories of the Eastern Health District, The Johns Hopkins School of Hygiene, Baltimore, Md.

tioned below have come to the personal attention of the writer, some in his own work, some as described to him by others.

1. Massive plate transfer, by which the entire surface of an apparently sterile plate is washed, the washings spread upon a second plate which is incubated, the entire surface washings of this plate then transferred to a third and so on until a growth appears. This invites contamination and a single minute contaminating colony will eventually appear as a mass of growth upon a subsequent plate. Any growths obtained by this method must obviously be regarded with the most extreme skepticism.

2. Rapidly repeated transfer from tube to tube through a long series not only invites contamination but selects from mixed culture a rapidly growing contaminant. This can appear finally in pure culture and may be regarded as a variant unless very critical examination is made of the organism and of the entire series of cultures.

3. Distribution of filtrates from flasks into large numbers of tubes followed by prolonged incubation also favors contamination and makes slow-growing contaminants appear to have developed from some principle in the filtrates. However, in considering the various techniques mentioned above, the error must be avoided of assuming that all growths resulting from them must be contaminants simply because a number of them may be.

4. Grinding of tissues and other material in mortars, even under the most extreme special conditions of asepsis, has been conclusively shown to be a source of serious error due to contamination from the environment.

5. Tissues taken from animals must obviously be regarded with suspicion because of the possibility of: (*a*) agonal or *post-mortem* invasion; (*b*) bacteria present in normal circulating blood or other body fluids; (*c*) alteration of an animal's resistance due to the injection of a pathogenic organism, so that other organisms, previously held in abeyance, enter the blood stream or tissues and may be confused with, or mistaken for, a modification of the original organism; (*d*) continued growth and post-mortem invasion of an animal, especially large animals like cats, rabbits and monkeys, the bodies of which remain warm for some time even in the ice-

box; (*e*) contamination during the autopsy of furry animals, which fairly teem with sporebearing and other kinds of bacteria which can enter the cultural field in spite of what are intended to be extreme precautions against this disaster.

6. Bacteria, especially acid-fast ones, may be found in smears of the tracheal and hilum glands and other organs of normal guinea pigs, thus giving the impression that tuberculosis in some form is present. The presence of a tubercle or some other definite tissue reaction is a better and safer criterion of this condition. The presence, from extraneous source, of saprophytic acid-fast bacteria in distilled water has led to error in searches for tubercle bacilli in smeared preparations prepared with such water.

7. Three kinds of organism, namely, sporeformers, diphtheroids and green-producing streptococci, found under conditions suggesting a variation or development from living principles in filtered materials or animal tissues, must be viewed with especial reserve because it has been repeatedly shown that these types are common environmental contaminants, practically ubiquitous and often very insidious in their means of entering the cultural field.

8. Some saprophytic and environmental bacteria are very delicate and fastidious growers and simulate pathogens in many respects. Their appearance only in blood or other special media and only after many days of incubation is often very suggestive of a delicate pathogen.

9. Some bacteria, gaining entrance to a plate at the time it is poured, will form minute, almost invisible colonies when the plate is stored for three or four days in the icebox. When the plate, supposedly sterile, is subsequently streaked, incubated and then, possibly, stored again, the bacteria from a single, original, minute, contaminating colony, may appear all over the surface of the plate. These new growths, appearing only after prolonged holding and without the plate's having been opened after purposeful inoculation, may not be connected with technical error unless special controls are used to eliminate this possibility.

10. The storage and re-use of plates once used but thought sterile is not permissible in research involving claims of variation of bacteria.

11. Media made from stale meat often contain, in surprising numbers, dead bacteria which were growing in the meat before it was used. The presence of these forms may lead to the idea that growth has occurred following inoculation with some filtrate or other material entirely innocent of any form of life.

12. Glass slides not properly cleaned may lead to error, especially in work with the acid-fast group.

13. The continued use of a pad of folded filter papers to dry slides, has led to error through the transfer of bacteria from one smear to another by the paper. Jars of stain for dipping slides may, after much use, contain bacteria which have washed off of the various slides stained.

14. Sediments derived from the disintegration of porcelain filters and from other sources may simulate living forms. They may appear as granules.

15. The presence of granules in any sort of biological material is not evidence that living organisms are present, no matter how much these granules may resemble preconceived ideas of what certain forms should look like. Granules resembling minute diplococci, rods, yeasts, filamentous bacteria and other forms may be found with the darkfield apparatus or in stained preparations from any tissue or albuminous material, whether previously inoculated or not. They will apparently increase upon incubation of uninoculated serum or blood bouillon or similar fluids, the material often becoming quite turbid after several days, due to changes in the colloidal status of the albuminous material responsible for their appearance. Granules of any sort must always be regarded as artefacts until proven otherwise.

16. Reports concerning details of the internal structure of bacterial cells must be viewed with caution because of the great difficulty of avoiding optical errors within the instruments used and in the bacteria themselves. It must be remembered that the curvature of these minute cells is very great so that refraction and diffraction effects are relatively enormous as compared with such structures as epithelial and liver cells.

17. Reports of morphological details based on camera lucida drawings and magnifications of 10,000 and 50,000 must obviously

be interpreted with the greatest reserve because present-day optical systems do not permit of such magnifications without great loss of clarity of outline and detail, if at all.

18. Plating and fishing of colonies, while generally useful, is not a sufficiently reliable method of purifying cultures in work involving bacterial variations. It is sometimes extremely difficult, if not impossible, to separate bacteriological species by this means. Single cell methods are much more reliable and, it would seem, furnish the only satisfactory means of solving our problems, but even such procedures as are at our disposal require very expert manipulation and may lead to error.

19. Many difficulties and sources of error are encountered in testing for fermentations, hydrolyses and various other biochemical changes. The medium may not support sufficiently vigorous growth of the organism reported upon; the incubation period may not have been long enough to permit the required changes to occur; the indicator used may not have been sufficiently sensitive to detect small amounts of acid if produced, or it may have been an indicator having its color range beyond the hydrogen ion concentration dealt with; the medium used may have been so highly buffered as to permit of no change in hydrogen ion concentration with the small amounts of acid sometimes produced; a test based upon the production of one substance from another, as nitrites from nitrates, may fail because of the the ability of the organism to attack the second substance as it is formed, so that unless parallel tests were run to control this, the test for nitrite production would give a false negative; loops made from wire other than platinum may affect determinations of hydrogen ion concentration where micro-colorimetric methods are used, since the alloy after repeated heatings, becomes coated with oxides which may impart a strongly alkaline reaction to substances not highly buffered. A large number of inadequacies of these types could be cited. Doubtless many others will occur to the mind of the reader. When considering reports of changes in any cultural or physiological characteristic of an organism, the methods used should be subjected to the most searching inquiry.

20. Organisms have been studied in one set of media and with

a given set of conditions at the outset of an experiment and later studied under conditions which have been modified in one or more particulars, possibly unknown to the investigator, and apparent changes found which have been reported as mutations but which really represented failure to recognize the true nature of the organism at the outset.

21. Filtration through porcelain and kieselguhr filters has been discussed so many times as a source of error that it seems hardly necessary to repeat these warnings. Nevertheless some of the factors involved may be mentioned at this time, and the first to be considered will be the time and the pressure used. High pressures applied for a long time may force bacteria through a filter which they would not ordinarily pass. High temperature of the filtered fluid will also facilitate passage of bacteria. The hydrogen ion concentration and composition (albuminous material, serum, plain broth or saline suspension, etc.) also affect passage of bacteria. The presence of serum or similar substances is apt to favor passage of bacteria, especially if large amounts (10 cc. or more per square cm. of filter surface) are filtered. The presence of even traces of oil or grease anywhere on the filtering surface facilitates passage of bacteria. Repeated use of a filter will result eventually in the passage of bacteria even though the filter may appear perfect when tested with air pressure. Baking of filters to clean, dry or sterilize them often renders them more permeable and frequently cracks them. Reports of the filterability of bacteria must therefore be regarded conservatively. Minute description of technical detail should accompany all such reports. The details should be regarded critically, each in turn.

22. Material reported upon after having been received from other laboratories has repeatedly afterward been found to have been contaminated or improperly labeled. Such material should never be accepted at its face value no matter where it comes from or how good it may seem. Whole pieces of research have been thrown under the shadow of doubt by failure to examine material from outside sources very carefully before commencing work with it.

23. When a mixture of two organisms, one of which grows rather

slowly, is lysed with bacteriophage, the slower growing one, hardly manifest, but now released from the reproductive and nutritive competition and not being susceptible to the bacteriophage used, may appear as a secondary growth and be regarded as a variant or mutant.

24. Observations made as long as six, seven or eight decades ago, when cited as corroboratory evidence, must be viewed critically because the cultural methods, reagents, optical instruments and knowledge of the subject, in general were, in many instances, subject to even more errors than our present systems, in support of which they are often quoted.

25. There is too great a tendency for the busy scientist to delegate a good portion or all of his technical work to a technical-assistant who, while conscientious and honest to the highest degree, may entirely lack the perception and the broad background of taining and experience necessary in intricate research problems. Such an assistant often fails to recognize error or new fact as the case may be and so does not eliminate or record it as the case may require.

Thus it appears that there are many known sources of error, some of them extremely subtle. Who knows what still unrecognized pitfalls evade detection in special problems?

The writer does not wish to be understood as defending or attacking any doctrine. By listing a few common sources of error he does not wish to imply that all reports not in accord with previous ideas should be regarded as the result of one or more of the listed errors. On the contrary, so much evidence of so unassailable a character has been presented recently that some previous notions are fading like the mists of night before an August dawn. However, caution must never be abandoned in any evaluation of bacteriological reports.

III. THE RELATION OF THE BACTERIAL VARIANTS OF KUHN TO THE CHIEF PHASES IN MICROBIC DISSOCIATION

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In the study of dissociative variation among bacteria it often becomes necessary to correlate in some orderly way the results and conclusions obtained by various investigators.

In microbic dissociation the chief culture phases at present recognized are the smooth (S), the mucoid (M), the rough (R) and the gonidial form (G). These phases are now being observed in an increasing number of bacterial species. They constitute the basis of pleomorphism with reference to both colony form and cell morphology; and they possess the greatest significance in relation to problems of biochemical behavior, antigenic structure, serological and immunological reactions, toxigenicity and virulence. Although we do not yet understand the biological significance of these culture phases in the life of the bacterial protoplasm, it is hardly safe to conclude that they possess no significance. Until such knowledge is forthcoming it appears satisfactory to apply to these phases simple terms of reference which correspond to some outstanding and easily observed morphological characteristic.

Kuhn, from his long study of variation phenomena, has also attempted to do this, but upon a somewhat different basis, employing in his case the morphology of the cell, rather than the characteristics of the colony. In this way Kuhn sets up five forms, the A, B, C, D and F. To permit of easier reference it seems desirable to attempt to relate, if possible, these culture types to the chief culture phases observed in dissociative variation.

Kuhn's B forms (Bakterien-Formen) comprise the ordinary rods, usually regarded as the "normal" forms of most bacterial species. These develop from the C forms.

The C forms (Kokken-Formen) are chiefly cocci or granular bodies, some of which are minute and filtrable. The colonies are small and sometimes microscopic. They arise from the B forms, according to Kuhn.

The D forms (dendritische Formen) are thick filaments and rods possessing root-like branches, perhaps produced by partial fusion. The morphology is often bizarre and they occur among the B and F forms.

The F forms (Faden-Formen) are chiefly threads and long filaments. They often produce mycelial net-works and develop, according to Kuhn, from the B forms.

The A forms (Pettenkoferia) are round or oval bodies, often attaining considerable size (six or seven micra). They multiply most often by fission, but are also believed to form large "cysts" in which "spores" are developed. Kuhn believes that these forms are produced as a result of the invasion of the bacterial cells by the spores of a Myxomycete, and that these spores actually constitute the bacteriophage.

Of these five forms, Kuhn regards as of special significance the B and the C types. These, he assumes, form the basis of all bacterial variation,—a dimorphism, rather than a pleomorphism in the sense previously indicated. The A, D, and F forms are apparently regarded as incidental.

In attempting to relate these five forms to the culture phases previously mentioned, it appears probable that Kuhn's B form is identical with the smooth (S). His C form seems definitely related to what have been termed the G forms. With this form at least some of the filtrable elements appear to be definitely associated. His F forms are clearly those of the R phase. As to his A and D forms, there exists no definite analogy with other defined dissociants.

In addition to the culture elements mentioned above, there is some evidence that there also exists in the life history of some bacterial species a form that may be called a virus-like form. It is filtrable through fairly fine candles such as the Chamberland L_3 and L_5; also through the N and W of the Berkefeld series. It is moreover apparently not visibly cultivable, at least on the

common laboratory mediums. If such a form exists, it might be called the virus-like or the V form, probably identical with the filtrable elements of the tubercle bacillus. Kuhn, on the other hand, denies that any such virus-like forms exist, but believes that the filtrable bodies are only the smallest of the C (or G) forms. As yet no conclusion can be drawn on this important question.

This problem of the culture phases has another important bearing. In dealing with such variants as the S, M, R, and G ele-

TABLE 1

Showing the relation of Kuhn's culture groups to the chief culture phases in microbic dissociation; also the relation of the latter to virulence in the case of pathogenic bacteria

KUHN'S GROUPS		DISSOCIATIVE GROUPS				TYPHOSUS DIPHTHERIA TUBERCULOSIS	FRIEDLÄNDER PNEUMOCOCCUS	STREPTOCOCCUS ANTHRAX
Symbol	Characterization	Symbol	Characterization					
	Not recognized	V	"Virus form"	+				
C	Coccus or granule	G	Gonidial		+			
B	Bacillus	S	Smooth			+		
	Not recognized	M	Mucoid				+	
F	Filaments or chains	R	Rough					+
D	Dendritic							
A	Pettenkoferia							

+ = assignment of virulence or toxigenicity.

ments, bacteriologists have commonly regarded as abnormal or degenerate all those cell entities that do not accord with the common cell type. Thus, in the typhoid species, the M and the R phases are regarded as abnormal or as "mutants." Since, in the anthrax bacillus, the rough (R) phase is most commonly observed, it comes about that the smooth (S) and the mucoid (M) phases become "abnormal." While, in the case of the Pneumococcus species and in Friedländer's bacillus, which most commonly appear in the mucoid (M) phase, the smooth (S) and the rough (R) would be the degenerate members.

The logic in this manner of interpreting the significance of certain phase variants, against which we can bring no other accusation except that they prefer to remain obscure, is difficult to understand. Is it justifiable to regard a culture phase as abnormal or degenerate merely because it is less commonly observed?

Furthermore, this issue carries another suggestive feature. If, among pathogenic bacterial species, it is sometimes the S, sometimes the M and sometimes the R phase that represents the most virulent or most toxic form of the organism (table 1), are we bound to conclude that there may not exist other species in which the G phase is the most dangerous; or still others in which the most virulent form may be the virus-like elements? This view, although as yet unproven, can scarcely be discarded with the apparent nonchalance characteristic of many recent writings on filtrable viruses and virus diseases.

IV. ATYPICAL ACID-FAST ORGANISMS

II. SOME OBSERVATIONS ON FILTRATION EXPERIMENTS

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In 1931 we published our totally unsuccessful attempts at demonstrating in pure cultures the presence of filtrable forms of the tubercle bacillus.[1] Although our results do not justify conclusions beyond the specifically stated conditions of our experiments, the fact should be stressed that some or all of the manifestations of the disease ascribed to a filtrable "virus" could be reproduced in guinea-pigs by what appeared to be a toxic factor in culture filtrates, and which was certainly not a living virus. In regard to technic, it was pointed out in that report that all so-called testing of bacterial filters is of problematical reliability and that, specifically, filters cannot be tested by bacterial species other than the one under scrutiny, and that any sort of testing before or after the experiment itself is unreliable. It was concluded that as far as normally virulent tubercle bacilli were concerned, the most satisfactory proof of the proper retaining power of a filter is the inability of the filtrates to produce tuberculosis in guinea-pigs.

Since that publication, an atypical acid-fast organism was isolated in this laboratory from the urine of a patient with renal tuberculosis. This organism was briefly mentioned in a recent publication[2] under group II. It will be described in full in a forthcoming report. Here, it will suffice to say that it is a fast-growing acid-fast organism which develops in perfectly smooth colonies, and which consists of small rods and many minute acid-fast and non-acid-fast granules. According to suggestions of other workers in this field, it was thought that this organism, on account of its many dust-fine granules, might be particularly suitable for

[1] Proc. Soc. Exper. Biol. and Med., **30**, 214.

[2] Amer. Rev. Tuberc., **24**, 73.

filtration experiments. Berkefeld filtrates from broth and agar cultures were prepared and were cultured in serial plate transplants according to Hauduroy's technic. On the third to tenth transplant, minute colonies made their appearance on the plates. These results were obtained with regularity with about 12 different filtrates. Eight separate strains were carried in pure culture for several months in order to ascertain some of their characteristics. Some of the properties that are common to the group are as follows:

1. They grow in small colonies, the diameter varying from 0.01 to 1.0 mm. The individual strains, however, show much less variation in colonial size than the group as a whole. The colonies are round and smooth (with the exception of one), either white and opaque or transparent. Daughter colonies, growing out of the margin of the mother colonies, are frequent; their diameter is one-fifth to one-tenth that of the mother colony.

2. They grow readily on all common media, better on solid ones than in broth; in the latter they produce slightly granular clouding and visco-granular sediments. They do not grow in the presence of gentian violet or malachite green in concentrations commonly used for the cultivation of tubercle bacilli.

3. The individual colonies show marked polymorphism: granules of different sizes are commonly associated with short, often granular, beaded or barred rods.

4. Stained by the Ziehl-Neelsen method, part of the organisms retain the carbol fuchsin. Usually the larger organisms are more acid-fast than the smaller ones. The acid-fast stain looks frequently more purplish than in tubercle bacilli. The relative number of rods and granules shows marked variation from strain to strain, as does the percentage of acid-fast elements. Four typical appearances of smears recurred with regularity:

   a. Small non-acid-fast granules.
   b. Acid-fast and non-acid-fast granules, most of them very small, but occasional ones having the diameter of two or three staphylococci.
   c. Acid-fast and non-acid-fast granules and rods; the former showing the variation in size as mentioned under (b); and the rods being beaded, clubbed or barred.

d. Relatively large acid-fast and non-acid-fast diplococcic forms.

With the exception of one strain, all strains showed, at times, the forms described under (a), (b) and (c). Only one strain showed the forms described under d. Which form developed could not be related to the media, to the age of the culture, nor to a cyclical change; with the exception that after repeated broth transfers, the granules increase in number and the rods finally disappear completely; but if the culture is, then, transplanted on agar, polymorphism recurs.

5. All strains are completely non-pathogenic for guinea-pigs; even when massive and repeated amounts are injected subcutaneously, no lesion beyond an occasional and ephemeral local swelling develops. Injected into the anterior ocular chamber of rabbits, they produce corneal clouding and slight whitish deposits on the iris which disappear without leaving a trace, within a few days.

6. They do not liquefy gelatin, with the exception of one strain.

7. Most of the strains do not ferment any of the common sugars, while one, the gelatin-liquefying strain, ferments glucose, levulose, galactose, lactose, maltose and sucrose. This fermentation is slow, producing acid, but no gas. This strain is the only one whose colonies have constantly definite rough characteristics.

The method by which these organisms were obtained, the regularity of their occurrence, the size of the colonies, their acid-fast characteristics and their polymorphism, all combined to create the impression that they were "G"-colonies. It must be obvious from the description that these organisms bear a close similarity to some of the bacterial forms that have been described as atypical or developmental forms of the tubercle bacillus. They contain morphological elements that seem to be eligible for the various terms applied to asexual and sexual cyclostages of acid-fast organisms.

In control experiments, sterile broth was substituted for bacterial filtrates, and the same serial plate transfers were made with broth. The results were identical with the ones obtained by transferring filtrates. The small colonies, described above, appeared

with equal regularity. It was then ascertained that our material, media, glass-ware, filters, did not contain these organisms, but that the air in the laboratory did.

Reference has been made to the report of other workers who have described bacterial forms as various cyclostages of acid-fast bacilli which have an embarrassing similarity with the air contaminators encountered in our laboratory. This is not done with the intention of disparaging such work; but it constitutes justification for scepticism and for demanding complete proof before such work can be accepted. It should be pointed out also, that our contaminators occurred with great regularity over a period of eight to ten weeks. The orthodox criteria for the recognition of contaminators are not adapted to the concept of bacterial polymorphism. New criteria must be developed. In recent work it is stated frequently that the regularity of a certain bacterial growth proves that it is not an incidental contamination; the findings reported here indicate that this argument is not valid.

The findings just reported, add nothing to the question whether tubercle bacilli have a filtrable phase, or developmental forms other than the classical Koch bacillus. They simply indicate some pitfalls that may be encountered in this type of work.

V. A HYPOTHETICAL VIEW OF BACTERIAL VARIATION

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Twenty years ago or more E. B. Wilson made the almost self-evident remark that heredity depends upon the integrity of cell division. This must apply equally to nucleated cells, to which he probably had reference, and to cells with a problematical nuclear structure like that of the bacteria. We have long been familiar with at least the basic mechanism of mitotic division and the elaborate procedure concerned in the division of the hereditary material of the parent cell and its dispersion to the daughter cells. Although genetical evidences exceed the present possibility of cytological analysis, genetics has not so far outrun cytology as to cause serious question of the correctness of the current explanation of mitotic processes. At the same time we are perfectly familiar with certain abnormalities in the division of nucleated cells as shown by both cytology and genetics. The examination of chromosome aberration, chromosome nondisjunction, crossing over and similar phenomena, in contrast to normal cell division and the resulting inheritance, constitutes a large part of contemporary cytological and genetical literature.

The integrity of cell division unquestionably controls normal heredity but it is equally evident that aberrations in cell division may result in irregularities in the transmission of hereditary factors.

The extent to which these considerations apply to such organisms as the bacteria is open to question. There are few or no pertinent experimental data. It is obvious, however, regardless of the various evidences of the presence or absence of a nucleus or nuclear material and without considering the indefinite question of sexuality, that in the ordinary or normal fission of the bacteria hereditary characteristics are equally distributed to the daughter

cells. This is certainly the simplest and most likely explanation of the uniformity of organisms ordinarily observed in a developing culture or in serial cultures of a bacterial species.

It seems not unreasonable to represent the normal fission diagrammatically as has been done in figure 1. If the absence of sexuality be assumed, for purposes of this thesis, the cells will be in the haploid condition. In figure 1 two unit characters are considered and represented by a square and a circle. As a first stage in the fission it is assumed that these genes, if they may be so styled, divide equally and subsequently the cell plate forms between the two halves. This results in two daughter cells of the same diagrammatical configuration as the parent cell. In the absence of any precise information concerning the structure

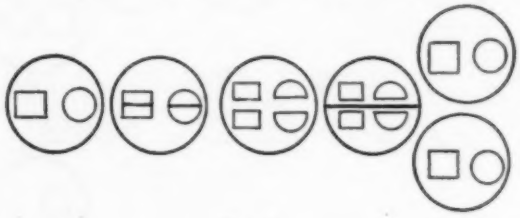

FIG. 1

or orientation of the cell constituents preceding or during fission it is unnecessary to consider whether the genes are in a nucleus, arranged in chromosomes or any comparable bodies, or freely distributed in the cell matrix. It is only essential, to the theory, that they separate in such a manner that the two halves are disposed on opposite sides of the cell plate and so are carried to the daughter cells. As long as this type of division of the hereditary characteristics, this type of division of all the genes in the cell, continues progeny will be like the parent; that is cultures or series of cultures will remain uniform.

If mitotic division occasionally fails to provide for equal distribution of hereditary material it seems equally likely that the, possibly simpler, process of fission will likewise fail in this normal function. In figure 2 a cell is represented, similar to that considered in figure 1, containing two genes. Preceding fission, one

of these divides and the halves separate, as in the previous case, but the second gene is represented as failing to divide, a condition possibly akin to chromosome nondisjunction, so that one daughter cell receives two genes and the second daughter cell a single gene. In figure 3, a second geometrical possibility is represented in which the gene indicated by a circle divides, as in the first stage of normal fission, but the two halves fail to segregate in such a way that the cell plate forms between them. As a result one daughter

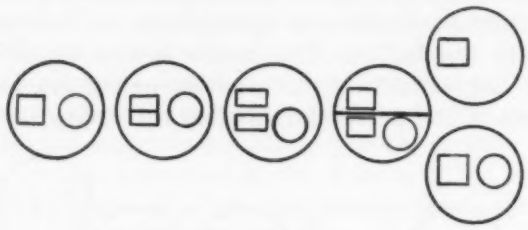

FIG. 2

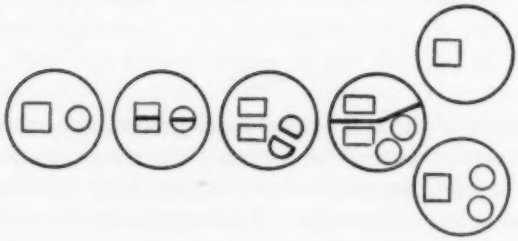

FIG. 3

cell will be without this gene and the second daughter cell will receive two such genes. If the possibility of either of these types of abnormal cell division be admitted, it then follows that the daughter cells which receive an abnormal quota of genes will, if they develop, differ from the parent cell in those characteristics transmitted by the genes in question. If the new types, arising as a result of abnormal fission, propogate subsequently by normal fission the new forms will be maintained.

In figure 4 a cell is represented as possessing four genes. This

may be considered the S form of a species known to produce at least three forms distinguished, for example, by colony structure. The squares might be regarded as representing the species genes, those genes which transmit the general species characteristics. The three circles represent the genes which transmit the S specific characteristic. For reasons which will be apparent below, the

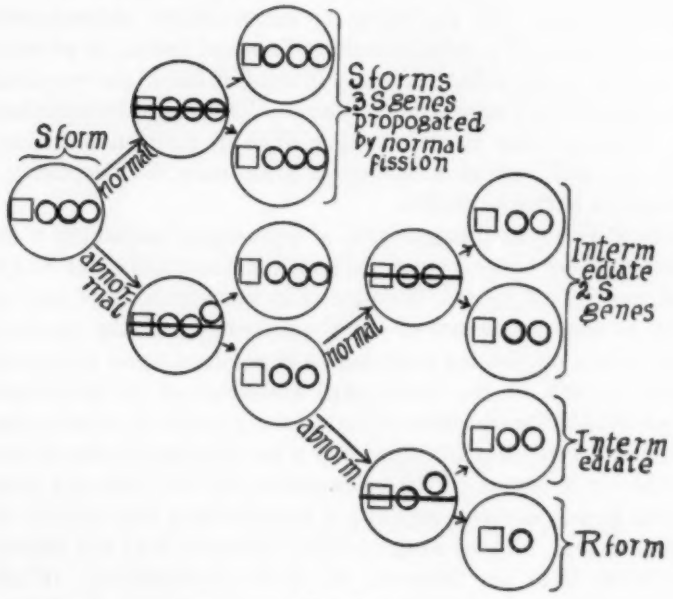

FIG. 4

S characteristics are represented as carried by three precisely similar genes, i.e., the S form is represented as resulting from the quantitative inheritance of a group of similar genes.[1] If an organism of this type is propagated by normal fission, similar to that described in figure 1, it will maintain its characteristic form indefinitely, as represented diagrammatically in the upper part of figure 4. (In figure 4 the supposed steps in the division

[1] This type of hypothesis has not been especially illuminating in the genetics of higher forms.

and segregation of the genes preceding the formation of the cell plate, shown in figures 1, 2 and 3, are omitted.)

If, in contrast to the normal, a fission occurs in which one of the three S genes fails to divide, one of the resulting daughter cells will lack one S gene, as indicated in the central portion of figure 4; this may constitute in intermediate type between the true S and the R form. So long as this new type is propagated by normal fission it also, will maintain its intermediate characteristics. The occurrence of a second similar abnormal fission, in which one of the two S genes fails to divide, will result in one of the two daughter cells receiving only a single S gene. This might be regarded as the R form. Any further irregularities in division resulting in cells with still further reduction in genes may, for simplicity, be treated as lethal or sterile.

The R form, in this analysis, is represented as simply a form which receives fewer S genes than do the intermediate or S types. This implies, of course, the idea that the synthesis of new material or the appearance of the characteristics of the species or variant in a developing organism is dependent upon the quantitative as well as the qualitative character of its inheritance. Three similar S genes are required to bring about the development of a true S type; the inheritance of a less number results in intermediate or R forms. This assumption and the supposed irregularities in cell division provides a hypothetical explanation of S to R variation. Such an explanation, however, does not allow, as is evident from the diagram, for R to S variation. In most species of bacteria studied from this point of view the R forms have proved to be relatively much more stable than the S, but at the same time have generally been found to be variable.

If such an irregularity in fission as suggested in figure 3 were to occur in an R form, constituted as just described, R to S variation would be produced. Figure 5 represents, at the top of the diagram such as an R, form propagating by normal fission and maintaining the R characteristics. Below, a fission is represented in which the one S gene divides and segregates, but in such a way that the two new genes remain on one side of the cell plate. As a result one daughter cell fails to receive an S gene, the second cell

receives two genes and, on the previously made assumption, becomes an intermediate form. A second similar irregularity will, as indicated at the bottom of figure 5, result in an R form and an S form. An examination of figure 5 will indicate that, if we had started with an S form, an R might have been derived by a similar

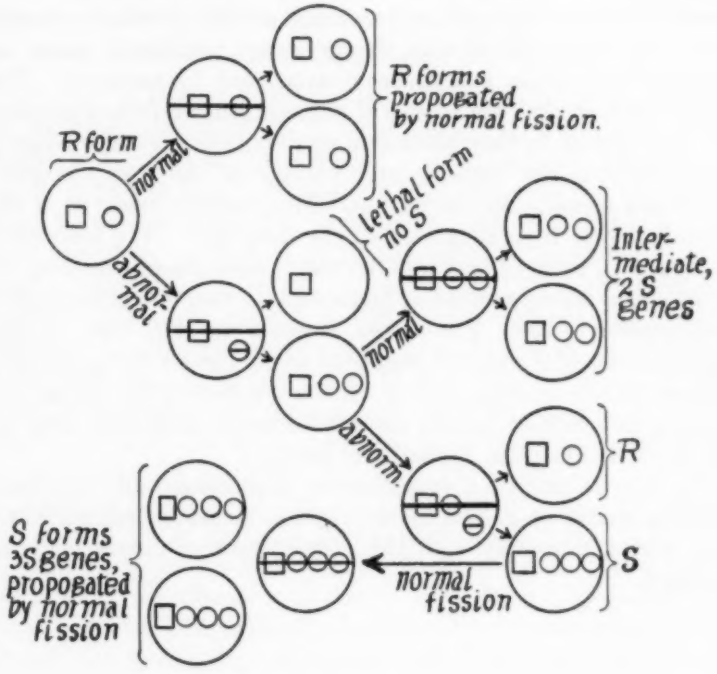

FIG. 5

series of irregular divisions. This type of explanation may therefore account equally well for R to S or S to R variation.

It is obviously unnecessary to the hypothesis to assume that any of these irregular cell divisions occur frequently. In the growth of an ordinary culture of bacteria there will be several million divisions and one or two abnormal divisions of the type just discussed, followed by normal fission will account for the ordinarily observed variations.

The degree of variability of a species, hypothetically, will depend upon the number of these accessory genes, genes which are not essential to development and propagation. It is implied in the diagrams that the group of species genes, represented by a square, and at least one S gene, are essential to survival and the maintenance of the species. Any irregularity in division which results in loss of any part of these must result in a lethal or sterile cell. On the basis of this theory many additional genes, as required to explain experimental data, may be assumed. The fact, however, that the species of bacteria studied from this point of view appear to vary according to a somewhat similar pattern suggests that the number and variety of these hypothetical accessory genes is not very large. The number, however, is obviously larger than suggested in the diagrams. The increasing evidence of independent inheritance of such characteristics, for example, as colony structure, pigmentation and virulence implies independent genes. The accessory genes are not, then, simply aggregations of S genes as suggested in the figures but genes concerned in the transmission of all the characteristics of the organism not carried by the essential species genes and may be readily fitted into the geometrical pattern.

As this is purely a hypothetical explanation of variation, nothing would be gained by attempting a comparison with current views, especially with the very attractive theory of cyclic changes.

VI. NOTES ON LIFE CYCLE PHENOMENA AND FILTRABILITY OF THE TUBERCLE BACILLUS

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Finality in science is an illusion. Irrespective of the import of the newer observations in bacteriology, no one today holds rigidly to the monomorphic theory of bacteria established in the youth of this science. My own part in this work has been confined to studies with the tubercle bacillus, supplementary or complementary to that of others whose work is omitted in this brief review for lack of space. Changes that I have observed are fundamentally not greatly unlike those reported with other species. The variations, therefore, seem to belong to all forms of bacteria and are not the result of a few isolated observations.

Notwithstanding the almost overwhelming array of new evidence, we must not be too hasty in modifying or overthrowing a stable order for an untried theory. For example, the majority of tuberculous infections can still be accounted for by the monomorphic theory of bacterial growth and reproduction. Likewise our knowledge of the Klebs-Loeffer bacillus apparently accounts for most of the disease diphtheria. The same may be said of other bacteria. Occasionally, however, there are peculiarities of growth and morphology of bacteria that even a tyro should not fail to observe and study. Such changes are the difference in virulence, colony formation, speed of growth, branching, lack of acid-fastness, formation of granules with appearances of sprouting forms, not to mention the so-called filtrable forms. Only a few of my observations concerning unusual phenomena will be cited, preceeding which I shall mention some controls.

Concurrent with the crucial experiments reported formerly,[1] I worked in a veritable wilderness of cultures, which were obtained

[1] Amer. Rev. Tuberc., **17**, 53; **17**, 77; **18**, 630.

in some relation to tuberculosis, either associated in cultures or in lesions. More than two hundred such strains were studied, some with three or more sub-groups. In respect to most of these strains I failed to show any relationship to the tubercle bacillus or to the disease, although it was equally true that I failed to prove that they were not related, except in their inability to become tuberculogenic. Many variable features were observed which established most of the strains as unstable and pleomorphic. For example, strains were observed to vary in morphology of both cell and colony. Wide chemical and biological differences were also noted from generation to generation, which prevented classification according to any recognized system. Although many seemed to be in intimate contact with the tuberculous process and were pathogenic, neither culture nor animal inoculation, including serial passage, could establish them as tuberculogenic unless they originated directly from a tubercle bacillus or a granule of a tubercle bacillus.

I mention this collateral work now because of its control value. These apparently barren experiments were interspersed among the successful experiments, affording valuable controls against spontaneous tuberculosis and against the finding of acid-fast bacilli in normal lymphatic structures. I may say parenthetically that neither in this large series nor in a large series recently studied, have we been able to find acid-fast bacilli in lymph nodes of guinea pigs, unless we were also able to relate them to contact with some form of the tubercle parasite. We have thoroughly studied the problem of spontaneous tuberculosis in this laboratory and know under what conditions it will appear. Our observations have been, that it will result in any laboratory if the guinea pigs are not properly dieted and are not kept away from contact with other tuberculous animals. A guinea pig in this respect is not different from a child. In view of this possibility, it cannot be denied, therefore, that any proof dependent upon animal passage alone is subject to serious criticism. Under the best of controlled conditions the disease is liable to develop, although it is true that it may be reduced to an insignificant minimum.

My experiences pertaining to variability of the tubercle bacillus, can best be illustrated in the short space allotted by a brief review of two aspects of the problem. First, observations of a life cycle different from the usual fission reproduction, and second, a summary of some new work on filtration.

For the first, I shall cite a few observations on a strain taken from a localized caseous pneumonia of a patient coming to autopsy (A175) at the Chicago Municipal Tuberculosis Sanitarium. The number of this strain was 159, and various subgroups included many strains of variable forms. Smears from the lung tissue revealed literally thousands per field of acid-fast bacilli, bearing at the ends or, most commonly, in the middle large purplish oblong granules measuring about 0.7 by 1.2μ. It was so nearly a pure culture that the problem of emulsification in salt solution and single cell picking became an easy matter. In addition, dark-field studies were made in salt solution and boullion suspensions for periods up to three days. In the dark fields, the granules within the bacilli appearing with a reddish refraction could be seen to divide first horizontally, then parallel to the bacillus, producing tetrads. Simultaneously they lost their red refraction and took on gradually a blue tint. No sprouting was observed as with a previously studied culture (no. 52) (American Review of Tuberculosis, January, 1928, plate I and plate II, figs. 1, 2 and 3), but stained smears revealed that many such changes as sprouting and the formation of comma-shaped, horseshoe-shaped and pessary forms had taken place in the course of reproduction.

The single cell pickings, however, were more convincing. Refractile granules were picked and inoculated subcutaneously into guinea pigs. One granular bacillus with one large granule and three small granules produced a slight and atypical tuberculous infection. Six and ten granules respectively produced a more typical tuberculosis, while an arbitrary dosage of about fifty in a larger series produced typical tuberculosis; smaller doses were all negative. This is, therefore, the first absolute proof that "Much's granules" will produce tuberculosis, although the granules observed by Much, no doubt, consist of a variety of forms differing in different circumstances.

Finally, a large number of single cells were picked both from the original and the first subcultures in an attempt to get pure cultures. After many attempts without results, one mold-like growth was obtained from the first subculture on Petroff's medium that produced a dry mucoid colony of a semi-acid-fast mycelial growth that killed guinea pigs with an atypical tuberculosis. Typical acid-fast bacilli were found in guinea pigs, but on artificial medium the culture gradually lost luxuriance and died out. This would ordinarily have been considered a mold contamination, but the mycelial threads contained acid-fast granules that seemed to warrant reporting.

The attempt to obtain a pure culture was repeated many times: finally we succeeded in isolating a colony from a single granule (and several from two or more granules). This is a typical human-type tubercle bacillus that has remained pure and unchanged for six years.

Since then, twenty-eight Maximow slide cultures with Long's medium have failed to demonstrate anything more than a sluggish microörganism growing slowly and forming granules but no "micro-gonidia" or other changes observed while in the original host.

These experiments go further to explain the difference of opinion that exists concerning such variability than any I can suggest. In the human host, we had a most unusual type of acid-fast bacillus with large granules. These granules seemed to change into tetrads under the dark field. They caused an atypical tuberculosis in guinea pigs in small doses. In a picked single granule in culture tube, a *Staphylococcus tetragenus* culture grew. Other cultures seemed to confirm this observation. Fixed and stained smears showed a number of acid-fast forms, apparently coming from granules in a number of ways. A complete series of transitions could be followed in both dark field and stained specimen. Our special senses must be trusted to a certain extent in such elementary observations. Certainly photographic lenses may be depended upon. Yet, when this culture became stabilized on an artificial medium, it grew from generation to generation with little change.

Another microörganism from autopsy no. 182 was even more interesting in that many semi-acid-fast forms could be caused to revert to acid-fastness and correspondingly develop tuberculogenic qualities. More recently a similar return to acid-fastness has been observed by a strain in culture.[3]

Although all the work reported is strongly presumptive, I have tried to present only as facts phenomena that could in some way be verified by photographic reproduction; reproduction by an unbiased artist; single cell culture studies; or animal inoculation experiments where the bacteriological findings were compatible with the pathology. I have tried to allow for appearances and illusions whenever there was doubt. The whole study reveals that certain strains of tubercle bacilli (if not all strains at certain times) have variations and modes of growth and reproduction in addition to the usual fission reproduction.

The filtration experiments have been repeated because of the numerous negative and indefinite reports recently in the literature and, particularly, because of the objections raised concerning contamination. My first experiments were conducted under conditions entirely acceptable to the older school of bacteriology regarding filtration. The filters were tested with fresh *B. coli* cultures for leaks (besides other precautions). If the filtrate did not produce growth in glucose boullion, surely there should be no question about any other *positive* results. There would be a more justifiable excuse to question negative results under such circumstances. In fact, filtration experiments are subject to such variable conditions (electrical charge on the filters, viscosity of medium, pressure, etc.) that it is a wonder any positive results are ever obtained when all aspects are rigidly controlled. As a technician, I had considerable public health bacteriological experience and my work was acceptable to several competent Laboratory Directors. In the same environment I had personally prepared, previously, more than a hundred litres of Cole's antipneumococcus serum without apparent contamination. Nevertheless, I felt that the work should be repeated on a larger scale

[3] Revista Medico-Chirurgica do Brasil, **37**, No. 9, Setembro de 1929.

with three types of Berkefeld filters nearly always in *duplicate*. In other words, I wished to conduct a "mass experiment." In the new experiments I was assisted by Miss Asya Stadnichenko, for whose aid I wish to express my appreciation. The material consisted of twelve specimens, including pus from cold abscesses; pus from non-tuberculous abscesses; macerated cultures of tubercle bacilli, macerated livers of fetuses from tuberculous mothers, and macerated animal organs.

The results seem to show that where no tubercle bacilli are present (as shown by controls) the animals all survive; where there is an avirulent tubercle bacillus, the animals also survive; where the culture is virulent there is an "asthenic" death in about two months, without tubercle, without acid-fast bacilli, and without the power to infect subsequent animals. With certain cold abscesses and other forms of septic material, there is likewise a "filtrate disease," but it is transmissible in series to other guinea pigs; in other cases there is a direct return to acid-fast bacilli and usually atypical tubercle formation, or there is an apparent return on one or two passages in animals. The work on three fetuses was negative for filter-passing forms, although one was positive in a control animal.

The theory of a "filtrate toxin" or a "bacillo-casein" cannot explain the phenomenon of transmissibility of the virus or the slow return to acid-fastness and tuberculogenic qualities. Neither does it account for the discrepancy between the results with virulent and non virulent strains. These can only be a filtrable part of the tubercle bacillus or a spontaneous tuberculosis in animals. The phenomenon seems to be occurring too regularly and too consistently in its time and manner of appearance to be spontaneous tuberculosis, especially since the controls fail to show a like change.

Finally, the "transmissible asthenia" seems to be entirely too common to explain on any such basis. As a theoretical explanation it may be thought of as a toxic death from very small gonidia or fragments of virulent tubercle bacilli that may go through an existence of their own, occasionally returning to acid-fastness but otherwise going on indefinitely until they run their course. Hered-

itary factors may not permit of the establishment of a stable form in their freshly liberated state. They may have to return to stability with some protective mechanisms or become extinct. Rapid passage may keep them in existence until stability is reached. The size of the forms may militate against any form of cell defense (tubercle or foci of inflammation), yet a toxic effect may be produced and maintained till the animal succumbs.

The question of filtrability of the tubercle bacillus is, therefore, not only open but seems to demand further experimental work. Especially should this be performed where there are any unusual phenomena in either the host or the parasite.

THE DISTINGUISHING CHARACTERISTICS OF LACTOBACILLUS ACIDOPHILUS

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Received for publication September 28, 1932

It requires only a casual examination of the literature to show that there is much confusion in regard to the differentiation of the members of the lactobacillus group. While bacteriologists who have worked with this group may have quite definite concepts of the various species it is difficult to translate those concepts into concrete terms which define a biological species. This is especially true of the acidophilus cultures, since they have been selected more on the basis of their ability to grow under certain prescribed conditions than because of compliance with taxonomic criteria.

Much of the confusion in bacteriological nomenclature is due to the use of a limited number of cultures in determining a basis for describing species and to reliance on characters which are inconstant and have little taxonomic significance. The natural grouping of bacteria is based on fundamental physiological characters which the members of these groups possess in common. Correlated with these are more superficial, and sometimes more obvious, characters which may permit an easy identification. In establishing a classification it is clear that the emphasis should be placed more on the stable basic characters and less on those which are superficial.

The isolation of cultures for identification is more or less a matter of chance and, if only a few are studied, there is always a possibility that these are not representative of the species as it occurs in nature. We have used as many cultures as the laborious nature of many of the determinations would permit.

The 103 cultures on which complete, or nearly complete, determinations were made were obtained through the courtesy of laboratories in different parts of the country. With the exception of a few cultures, presumably bulgaricus, and cultures of dental and vaginal origin, nearly all were labelled acidophilus. The origins of these cultures in so far as they could be traced are given in table 1.

Microscopically all the cultures were Gram-positive, non-sporulating, non-motile rods, varying in size when grown in milk from short rods to long filaments. Acid was always produced in milk with no evidence of gas formation. Colony form varied

TABLE 1

Source of cultures

	NUMBER OF CULTURES
Human vagina	9
Intestine, human or rat	23
Dental	24
Acidophilus preparations	13
Unknown	34
Total	103

from the fuzzy and filamentous to the regular smooth sand-grain type with all intermediate gradations. Before use each culture was plated on tomato peptone agar and incubated at 37°C. in an atmosphere of 10 per cent carbon dioxide. After forty-eight hours well isolated colonies were examined with the 16 mm. objective. Film preparations from these colonies were stained by the Gram method and examined. If no evidence of contamination was then apparent the remainder of the colony was transferred to sterile litmus milk, subcultures of which were used later for the experimental work. These cultures were replated at frequent intervals in order to eliminate possible contamination. Throughout every experiment each control and test culture was examined microscopically before and after incubation to verify purity and positive growth.

In this study the following cultural and physiological characters have received attention: total and volatile acid production, nature of the volatile acids, form of lactic acid, limiting undissociated lactic acid, sugar fermentations, carbon dioxide production, limiting growth temperatures, limiting phenol and indol concentrations, and surface tension relationships.

TOTAL AND VOLATILE ACID PRODUCTION

Except for carbohydrate fermentations most of the previous biochemical studies upon the lactobacilli have been limited to the principal non-volatile product of fermentation, viz., lactic acid. Very few published data are available concerning the volatile acid production of these organisms. Heinemann and Hefferan (1909) found that *B. bulgaricus* produced about 6 per cent of volatile acid in milk. Barthel (1913) studied 10 aciduric bacteria isolated from a variety of sources, and found that the amount of volatile acid formed, expressed in per cent of the total acids, varied in the different cultures from 1.1 to 7.1 per cent. Hart *et al.* (1914) studied the action of certain bacteria concerned in the ripening of Cheddar cheese. When 2 strains of *Bact. casei* were incubated for seven months in milk most of the total acid then present was volatile. Kopeloff (1926) refers to unpublished analyses by Zoller in which the total volatile acid approximated 5 to 10 per cent of the combined volatile and non-volatile acids. Apparently this work was limited to a small number of authentic acidophilus cultures.

In our study of volatile acid production the following procedure was adopted: 260 cc. of Swiss cheese whey was inoculated with 1 cc. of a forty-eight-hour milk culture and incubated at 37°C. After six days, 10 cc. of the culture was withdrawn and titrated for total acidity with 0.1 N sodium hydroxide. The remainder was made acid to Congo red paper with phosphoric acid and distilled with steam until 1 liter was obtained. An aliquot of this was titrated with 0.1 N NaOH and the remainder made alkaline to phenolphthalein with NaOH and evaporated to dryness over a steam bath. The residue was collected in 10 cc. of distilled water and used for the identification of the volatile acids.

The volatile acid production of our cultures is represented graphically in figure 1. The strains are arranged according to the proportion of total acid which is volatile. It is evident that most of the cultures are concentrated in a small volatile acid range. In 50 per cent of the cultures the percentage volatile of the total

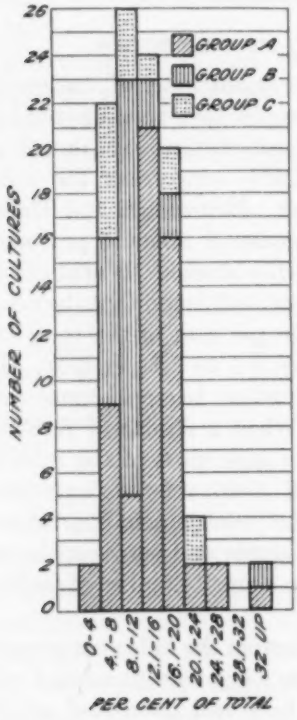


FIG. 1

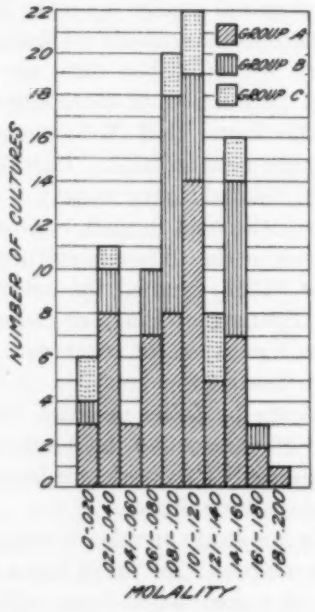


FIG. 2

FIG. 1. DISTRIBUTION OF CULTURES ON THE BASIS OF VOLATILE ACID PRODUCTION
FIG. 2. DISTRIBUTION OF CULTURES ON THE BASIS OF LIMITING UNDISSOCIATED LACTIC ACID CONCENTRATION

acid was between 10 and 20; in 38 per cent between 4 and 10. In only 8 per cent of the total cultures was the volatile acid less than 4 or over 20 per cent of the total acid. The two strains in which over 30 per cent of the total acid was volatile were obviously out of place in a collection of acidophilus cultures. The

compactness and single peak character of the distribution curve is evidence of a certain uniformity in the amount of volatile acid produced. Upon the basis of volatile acid production therefore these cultures are relatively homogenous and can not be divided into well defined groups. As will be shown later, however, the quantitative production of volatile acid correlates well with many other characters and lends itself readily to a classification based upon certain of these criteria.

The total acidity produced in these cultures varied from 0.227 to 2.70 per cent, calculated as lactic acid. In over 75 per cent of the cultures the titratable acidity was between 0.5 and 1.5 per cent. Approximately 20 per cent produced less than 0.5 per cent acid while very few yielded more than 1.50 per cent total acid. The low acid group is somewhat larger than might be expected and to some readers the absence of appreciable numbers of high acid formers in a collection of this size may occasion some surprise.

The titratable acidity and total volatile acidity increased only slightly after the first four days. That the ratio between the total acidity and total volatile acidity is practically constant is indicated by the fact that determinations on new samples after the lapse of one year showed only small variations from those originally found.

NATURE OF VOLATILE ACIDS

The positive identification and estimation of individual members of the volatile acid series is not readily accomplished. The partition method recently proposed by Werkman (1930) does not differentiate accurately between formic and acetic acids both of which were formed in our cultures. The Duclaux method of fractional distillation, though admittedly inexact and subject to limitations, is capable nevertheless of yielding valuable comparative information and was therefore used in this study.

The alkaline residue obtained in the quantitative estimation of volatile acids was made acid to Congo red by the addition of concentrated phosphoric acid; the solution was then diluted to 110 cc. with distilled water and fractionally distilled in 20 cc. increments according to the method of Duclaux (1900). The separate portions were then titrated and the results calculated as percentages

of the distillate in 100 cc. The titration constants for the five fractions showed a remarkable constancy for the entire group of cultures. These figures are omitted to economize space. Over 90 per cent of the cultures upon Duclaux distillation gave distillation constants indicating a mixture of the same acids in the same ratio. This surprising similarity of the titration figures affords unmistakable evidence that the cultures produced the same combination of volatile acids. It is equally clear that this phase of their metabolism offers no satisfactory basis for differentiation. The distillation constants fail uniformly to coincide with any two acid combinations in the Duclaux table. This lack of correspondence with known solutions suggested a complex mixture of three or more acids. The titration constants most nearly approached those for formic, acetic and butyric acids and as the constants for formic and acetic acid fall very close together it seemed likely that the volatile mixture was made up of these three acids. As this evidence was only presumptive, positive methods of analyses were then sought. The Jones method for the identification of formic acid based upon the reducing action of the aldehyde radical was used for a time but later abandoned when it was found that the sterile uninoculated whey medium contained non-acid volatile reducing substances. The mercuric oxide test for formic acid which depends upon the solubility and stability to heat of mercuric formate was finally chosen to demonstrate the presence of this acid. This test was applied as follows: The volatile acid distillate was warmed to 40°C. with an excess of HgO and filtered through paper. The filtrate was then boiled in a water bath which decomposed the mercuric formate yielding a heavy grey precipitate of metallic Hg. Every culture gave a positive reaction to this test which was accepted as positive proof of the presence of formic acid in these distillates. The identification of the other volatile acids was accomplished by repeated fractional distillation of the volatile acid mixture and observation of the boiling points. In order to provide a sufficient quantity of the acids for this purpose all the individual volatile acid distillates were combined. A composite 3-liter sample was extracted with ether and the aqueous and ether phases were separated by means

of a separatory funnel. The ether was then distilled off and the water-free volatile acid residue fractionally distilled until relatively pure samples were obtained. The odor, relative quantity, and boiling points of the samples indicated the presence of formic, acetic and butyric acids in the proportion of 6:3:1. These values are necessarily approximate and probably vary at least 15 or 20 per cent with the individual cultures. These results, confirming as they do previous deductions, constitute, we believe, satisfactory evidence of the identity of the volatile acids generated in these cultures.

FORM OF LACTIC ACID

It seems probable that whenever a cell produces lactic acid, it produces both the dextro and the levo form, at least ultimately. The ratios between the quantities of the two forms vary with a considerable number of factors, among which have been included kind of sugar fermented, temperature of incubation, the species of lactic organism used, and the presence of organisms which do not produce lactic acid (Pederson, Peterson, and Fred (1926) and references which they give to earlier work). Pure cultures grown under the same cultural conditions produce consistently the same ratio of the two forms of acid and it is for this reason that this method was tried as a basis for differentiation of species in the acidophilus group. Kopeloff and Bass (1927) have reported that three cultures which they designated *L. acidophilus* gave dextrorotatory acid in excess of levo.

The method used in our work was that of Pederson, Peterson, and Fred (1926), with modifications suggested by Dr. Fred.

In order to obtain sufficient acid for analysis the following procedure was adopted: Swiss cheese whey was concentrated in a vacuum pan until a large part of the lactose crystallized out. This sugar was removed by centrifugation and the liquor restored to the original volume with water. The final product was consequently normal whey with a greatly reduced sugar content. Two liters of this reconstituted whey were inoculated with 1 cc. of a forty-eight-hour milk culture and incubated at 37°C. After six days the culture was made acid to Congo red paper with phos-

phoric acid and distilled until all of the volatile acids were liberated. This residue was further concentrated to a volume of about 100 cc., maintained acid to thymol blue with phosphoric acid and extracted continuously for seventy-two hours with either isopropyl ether or a mixture of isopropyl and ethyl ethers. Approximately 25 cc. of water was added to the extract and the ether removed by distillation. The water solution was then made up to 100 cc. and a 5 cc. aliquot titrated to determine the quantity of extracted acid. The remainder of the solution was boiled for ten minutes or more with an excess of alkali-free zinc carbonate. The excess carbonate was removed by filtration and the filtrate evaporated on a steam bath until crystallization began. Ethanol was then added to give a concentration of 50 per cent, the solution well stirred and kept over night at 10°C. The zinc lactate was then filtered off, washed with 95 per cent ethanol several times, then with ethyl ether, air-dried and bottled.

In order to determine whether the lactate was optically active or inactive, it was necessary to determine the water of crystallization. The active salt has two molecules of water of crystallization or 12.89 per cent; the inactive, three molecules, or 18.18 per cent. The percentage of inactive salt may be calculated from the formula

$$100 \times \frac{\text{per cent } H_2O - 12.89}{18.18 - 12.89}$$

A 2-gram sample was weighed in a moisture dish, dried over $CaCl_2$ to constant weight and the water of crystallization driven off by drying to constant weight in an oven at 110°C.

The results obtained were very clear cut as a basis for sharply grouping the 96 cultures examined. For 69 cultures, the determinations indicated practically pure inactive salt which denotes the presence of the acids in equal quantities. Of the remaining 27 cultures, 26 contained from 65 to 95 per cent of active salt in excess and one contained 52 per cent. In all 27 cases the rotations of the active salts were levo; therefore, the acid in excess was dextro-rotatory.

LIMITING UNDISSOCIATED LACTIC ACID CONCENTRATION

In the earlier years of bacteriology, the cessation of fermentation by acid-producing organisms was assumed to be determined by a limiting gross concentration of acid. More recently, with a better understanding of hydrogen-ion concentration and buffer action, the actual limiting factor was believed to be the concentration of the hydrogen ions. Hydrogen ion concentration in a medium is a function of concentration of titratable acid and of characteristic intensity and concentration of buffer substances present. Undoubtedly hydrogen ion concentration is of itself the limiting factor under usual conditions. However, still more recently (see review by Kolthoff, 1925) the undissociated forms of acids have been shown to be inhibitory. The concentration of the undissociated form is a function of the hydrogen ion concentration and of the total concentration of the acid present.

Van Dam (1918) showed that in *S. lactis* cultures in whey to which differing amounts of neutral sodium lactate have been added, fermentation ceased at different pH values. The calculated concentration of undissociated lactic acid was shown to be the constant limiting factor under these conditions. This limiting concentration varies with the medium used, but appears to be a reproducibly constant characteristic of a species of organism, since Rogers and Whittier (1928) were able to check Van Dam's values for *S. lactis* in whey. It was hoped that limiting concentration of undissociated lactic acid might serve to differentiate the lactobacilli from one another. Differences in limiting values for our laboratory cultures were found of sufficient magnitude to justify making the necessary determinations on our collection of lactobacilli.

The equation used in the calculation is the following:

$$(\text{H lactate}) = \frac{(\text{total lactate})}{1 + \frac{K}{\alpha(\text{H}^+)}}$$

in which K is the dissociation constant of lactic acid, 14.7×10^{-5}, α is the degree of dissociation of sodium lactate, varying from 0.76 to 0.80 for the concentrations used; and the brackets

signify concentrations of the substances bracketed in mols per liter. The determinations needed are of total lactate concentration and of hydrogen-ion concentration.

To three flasks containing 100 cc. of Swiss cheese whey were added respectively, 2, 3 and 4 cc. of approximately 6 M solution of neutral sodium lactate. The flasks were plugged with cotton and autoclaved for twenty minutes at 120°C. After being cooled, each flask was inoculated with 0.5 cc. of a vigorous culture of the organism being tested. The flasks were then incubated at 27°C. After four days, the cotton plugs were removed and sterile rubber stoppers inserted in the necks of the flasks. Cotton plugs permit evaporation of the whey which affects the final results. After at least two weeks' incubation, the hydrogen-ion concentration was determined by means of the quinhydrone electrode and the total lactate concentration by the method of Friedemann, Cotonio, and Shaffer (1927). The sample was filtered and the filtrate used directly for the lactate determination without other treatment. The undissociated lactic acid concentrations calculated from these determinations usually agreed within five units in the third decimal place.

Figure 2 shows the distribution of these cultures, depending upon their limiting concentration of undissociated lactic acid. As may be seen, three rather distinct groups are indicated, one for a very low concentration of acid varying from 0.020 to 0.040 moles per liter, one between 0.060 and 0.120 and one above 0.140. Thus, these cultures are not checked uniformly by a given concentration of the undissociated acid but apparently represent three distinct groups in which the limiting concentration of undissociated lactic acid varies within a considerable range.

Limiting pH values for all the cultures were determined in skim milk. These values correlated with limiting undissociated lactic acid as might be expected, but very grossly. There was no noticeable correlation with other determined characteristics and consequently no report is made of the results obtained.

FERMENTATION OF CARBOHYDRATES

The value of carbohydrate reactions in the classification of aciduric bacteria is a question upon which there is no general

agreement. Rahe (1918) proposed a classification of these organisms based upon the fermentation of glucose, maltose, lactose, sucrose and raffinose, Kulp and Rettger (1924) differentiated *L. acidophilus* and *L. bulgaricus* by their ability to attack maltose, sucrose and unheated levulose. Albus and Holm (1926) declared that sugar reactions were unreliable for the separation of these organisms. Day and Gibbs (1928) also failed to confirm the results of Kulp and Rettger and concluded that the fermentation of maltose, sucrose, and levulose does not offer a means of distinguishing between *L. acidophilus* and *L. bulgaricus*.

The fermentation reactions of Döderlein's vaginal bacilli were compared with those of the intestinal or acidophilus types by Joetten (1922), Lash and Kaplan (1926), Thomas (1928), and others. In general, no consistent differences were observed. Dental and intestinal strains have been compared directly by Morishita (1928); Rosebury, Linton, and Buchbinder (1929); Hadley, Bunting and Delves (1930); Howitt (1930); and many others. Of these, only Morishita found significant differences in nature of the carbohydrates utilized.

The basic medium used in our work consisted of casein-digest broth prepared from C. P. casein by the tryptic digestion method described by Kulp and Rettger (1924). Ten per cent solutions of the test substance were made up in distilled water. Levulose solution was sterilized by filtration through a Berkefeld filter candle, while all the other sugar solutions were sterilized by steam under pressure. These were added aseptically to the sterile basic medium in sufficient quantity to yield a final concentration of 1 per cent. In order to furnish more favorable growth conditions each 10 cc. of the medium also received 3 drops of sterile horse serum. This serum was heated to 60°C. for one-half hour prior to its use in the maltose solutions (TenBroek 1920). The final reactions of all the media were between pH 6.8 and 7.1.

The inoculum was prepared by growing the culture at 37°C. in casein digest broth containing 0.1 per cent glucose. At the end of twenty-four hours the organisms were sedimented by centrifugation, the supernatant fluid was poured off, and the cells were resuspended in sterile distilled water. This process was repeated several times, after which the washed cells were diluted

with sterile water to an arbitrary density comparable with that of a standard suspension of barium carbonate. One-tenth cubic centimeter of this suspension was used to seed 10-cc. portions of the test medium. After ten days' incubation at 37°C. the hydrogen-ion concentration was determined colorimetrically by the method described by Brown (1924). A distinct change in pH from that of the inoculated sugar-free control was accepted as evidence of carbohydrate utilization.

The study of the fermentative activity of these cultures was made upon the following substances: Lactose, maltose, sucrose, unheated levulose, raffinose, salicin, mannitol. They are arranged in the order of availability. With one or two exceptions all the cultures fermented lactose; sucrose, maltose, and unheated levulose were utilized with very few exceptions. Raffinose was attacked by about 60 per cent of the cultures and salicin by more than half. Acid was produced from mannitol by approximately 29 per cent. It would appear that these reactions remain fairly constant from the fact that 30 of the cultures retested after the lapse of one year showed only slight variations in the final pH value. In none of these cultures was the ability to ferment a given substance either lost or acquired.

CARBON DIOXIDE PRODUCTION

Carbon dioxide appears as one of the end products in the decomposition of carbohydrates by most bacteria. It may be found in exceedingly small quantity or occur as one of the major products of the fermentation. This particular phase in the metabolism of the lactobacilli has received very little attention in the literature. Eldredge and Rogers (1914) isolated and studied a large number of cultures from Emmental cheese. While no attempt was made to identify the organisms, the recorded cultural and physiological characters indicate that many belonged to lactobacilli of the casei-bulgaricus group. Approximately 95 per cent of the lactose-fermenting rods produced carbon dioxide in varying amounts in sugar-free whey. Sherman (1921) studied the CO_2 production of 38 cultures of aciduric bacteria. This collection included strains of *L. acidophilus*, *L. bulgaricus*, and

L. bifidus, as well as unidentified cultures from intestinal contents, silage, milk, and cheese. Many of these organisms were found to produce significant amounts of CO_2 and the bulgaricus types produced less than the intestinal forms. Fred, Peterson, and Stiles (1925) found that the granulated lactic acid bacteria from cereals formed little or none of this gas in yeast broth containing glucose, fructose, or lactose.

Carbon dioxide was measured quantitatively by the following method: Twenty cc. of unfiltered Swiss cheese whey was steril-

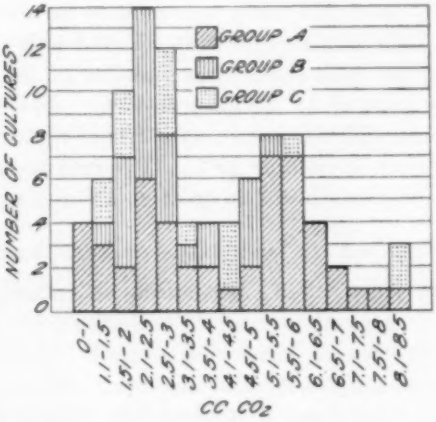


FIG. 3. PRODUCTION OF CARBON DIOXIDE GAS

ized in Eldredge tubes. These were immediately cooled to 37°C. and inoculated with 0.1 cc. of a 48-hour milk culture. Ten cubic centimeters of $Ba(OH)_2$ were then measured into each tube to absorb the CO_2, after which the cultures were incubated at 37°C. Absorption of atmospheric CO_2 was prevented by sealing the upright tubes after inoculation. At the end of five days the excess of $Ba(OH)_2$ was titrated with 0.1 N oxalic acid and the CO_2 calculated from the difference. The quantity of CO_2 was expressed in cubic centimeters of N/10 $Ba(OH)_2$ neutralized.

Figure 3 shows the range of CO_2 produced in these cultures and their distribution in the different zones. As may be seen the

cultures fall largely into two general groups, those forming 4 cc. or less and those forming from 4 to 7 cc.

LIMITING GROWTH TEMPERATURES

The temperature relations were established by heavily inoculating litmus milk tubes and holding them in incubators at 10°, 15°, 20° and 30°C. and in water baths at 37°, 40°, 43°, 46°, 48°, 50° and 52°C. Growth was determined by observing changes in the color of the litmus. At the low temperatures the cultures were held for two months. The optimum growth temperature was between 37° and 40°C. and was uniform for the entire collection. None of the cultures grew at 52° and only a very few at 50°C. The upper limit for a large number of the cultures was 46°, and many grew at 43° and not at 46°C. In the lower range there was a sharp differentiation, with 79 per cent failing to show evidence of growth in milk at 20°C. The remainder grew at 20° and many of these continued to grow at 15° and even 10°C.

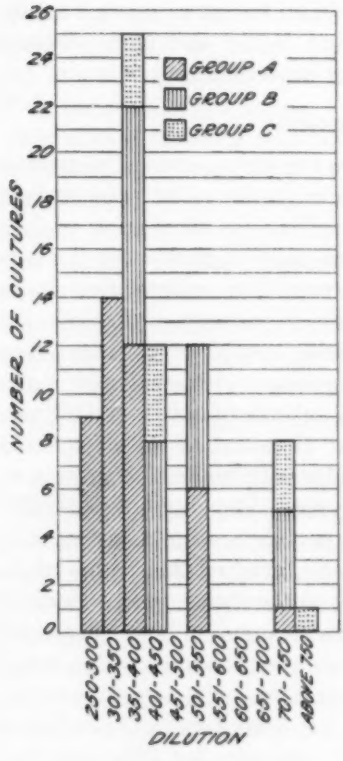
LIMITING PHENOL AND INDOL CONCENTRATIONS

The work of Kulp (1929) introduced an entirely new means of separating *L. acidophilus* and *L. bulgaricus*, based upon their relative sensitiveness to indol and phenol. Concentrations of these substances completely inhibitory to *L. bulgaricus* exercised no restraining action upon *L. acidophilus*. While his study was limited to comparatively few cultures, the results seem to justify the conclusion that typical *L. bulgaricus* and *L. acidophilus* may be differentiated by the determination of their tolerance for indol and phenol. As both of these substances are present in appreciable amounts in the intestinal canal of man and animals, it is not illogical to suppose that they may play a rôle in determining the implantability of lactobacilli.

The plate method and technique described by Kulp (1929) was exactly duplicated in all details. Tomato peptone agar (Kulp 1927) was the nutrient medium and the test reagents were Kahlbaum, C.P. quality.

The relative tolerance of these cultures to phenol and indol is shown in figures 4 and 5. The distribution curve for phenol

(fig. 4) reveals three rather distinct peaks indicating that the cultures were not alike in their response to different concentrations of this substance but may be divided on this basis into two



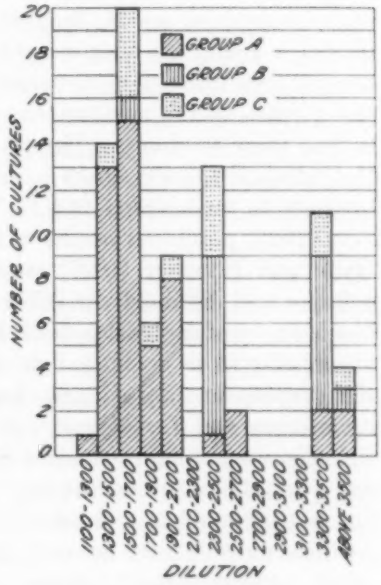


FIG. 4 FIG. 5

FIG. 4. DISTRIBUTION OF CULTURES ON THE BASIS OF THEIR ABILITY TO GROW IN THE PRESENCE OF PHENOL

FIG. 5. DISTRIBUTION OF CULTURES ON THE BASIS OF ABILITY TO GROW IN THE PRESENCE OF INDOL

or more groups. About 75 per cent of the cultures were inhibited by a phenol concentration of 1:450. Sixteen per cent grew between 1:500 and 1:550, while the remainder constitutes a small

group which grew in concentrations as high as 1:750. The limiting concentrations of indol (fig. 5) present a similar picture; the distribution of cultures as in the preceding figure shows two or three distinct divisions. The first group comprises over 60 per cent of the cultures, all of which failed to grow above 1:2100. The other two groups are somewhat larger and more distinct than was true in the limiting phenol concentrations.

SURFACE TENSION

Albus and Holm (1925, 1926) were the first to study the growth of *L. acidophilus* and *L. bulgaricus* in media of lowered surface tension. Using the drop-weight method for measuring surface tension they found that *L. bulgaricus* did not grow when the surface tension of the medium was depressed by sodium ricinoleate to less than 40 dynes. The authors in consequence concluded that surface tension offers a valuable means of distinguishing between *L. acidophilus* and *L. bulgaricus*. Kopeloff and Beerman (1927) published results which substantiated these findings. Hyde and Hammer (1927) studied 12 so-called *L. acidophilus* cultures and obtained variable results with respect to growth in media at a surface tension of 37.4 dynes. Day and Gibbs (1928), attempting to throw more light on surface tension as a factor in the development of aciduric bacteria, studied 20 strains of *L. acidophilus* and *L. bulgaricus* from authentic sources. Sodium ricinoleate was found to exert a toxic action upon both bulgaricus and acidophilus strains but they concluded that this action could not be employed as a means of identifying the two organisms. As these authors have pointed out, the weak link in the evidence which regards surface tension as a criterion for differentiating between *L. acidophilus* and *L. bulgaricus* is that different soaps used as depressants do not give uniform results, indicating that the chemical properties of the depressant rather than surface tension were probably the significant factors in earlier work.

In view of the conflicting nature of the evidence upon this subject it seemed desirable to study the growth of our cultures in the presence of commonly used surface tension depressants. The yeast lactose brom-cresol-purple medium employed by Albus

and Holm (1926, 1928) was chosen for this work. Neutral sodium oleate (Merck) and sodium ricinoleate (Merck) were used as surface tension depressants. Surface tension was measured by the duNoüy tensiometer at 25°C. before and after sterilization of the media; 0.2 cc. of a forty-eight-hour milk culture was used to inoculate each 8 cc. of medium. Positive growth was recorded with any change in the color of the indicator after incubation at 37°C. for forty-eight hours.

When sodium oleate was used to depress the surface tension of the media, all of the cultures grew at 34.5 dynes per centimeter. With sodium ricinoleate as the depressant, practically all the cultures grew at 38 dynes. Occasionally a culture would fail to grow initially at this concentration but subsequent cultivations were always positive. Unless we are to make the highly improbable assumption that our collection contained no organisms of the bulgaricus type, it would appear that the action of these surface tension depressants can not be used as a means of differentiating *L. acidophilus* and *L. bulgaricus*.

CORRELATION OF CULTURE CHARACTERS

The hopelessness of classifying these cultures in any arrangement approaching the usual dichotomous scheme will be realized on examination of table 2. In this table there are, if we include the combinations made possible by incomplete determinations, 47 groups. This number could be increased indefinitely by adding more characters. It is only by gathering the cultures into groups having as many characteristics in common as possible that we can hope to arrive at any order.

The most clear-cut division which we have is on the basis of the nature of the lactic acid produced. The distinction between the cultures forming inactive acid and those forming both inactive and dextro acid is sharp and is based on a fundamental physiological reaction. The test of the soundness of this division will be found in the number of other characteristics which correlate positively or negatively with the type of lactic acid.

In making up these groups we have included a few cultures on which the lactic acid was not determined but which we considered

TABLE 2

Characteristics of 103 Lactobacillus acidophilus cultures

GROUP	CULTURE NUMBER	LACTIC ACID		LIMITING UNDISSOCIATED LACTIC ACID			CO_2		GROWTH AT 20°C.	FERMENTATION		
		Inactive	Inactive + D	Below 0.050	0.051–0.130	0.131–0.200	Below 4 cc.	Above 4 cc.		Raffinose	Mannitol	Salicin
1	104	+		+			+		+	+	+	+
2	91	+		+			+		−	+	+	−
3	28, 21	+		+			+		−	+	−	−
4	6, 12, 78	+		+			+		−	+	−	+
5	5, 9	+		+			+		−	−	−	+
6	88	+		+				+	−	+	+	+
7	60	+		+			?	?	−	+	−	−
8	62, 65	+			+		+		+	−	−	+
9	23, 32, 33, 35, 106	+			+		+		+	−	−	−
10	11, 15, 24, 44, 81, 82, 93	+			+		+		−	+	−	+
11	76	+			+		+		−	+	−	−
12	79	+			+			+	+	+	+	+
13	73, 77	+			+			+	+	+	−	+
14	10, 36, 42, 47, 55, 85, 87	+			+			+	−	+	−	+
15	27, 31, 34, 38, 39, 40, 43, 46, 48, 98	+			+			+	−	+	−	−
16	2	+			+			+	−	−	−	−
17	16, 13, 53	+			+		?	?	−	+	−	+
18	3, 50	+			+		?	?	−	+	−	−
19	101	+			+		+		−	+	+	+
20	99	+				+	+		+	−	+	+
21	74	+				+	+		−	+	+	+
22	1	+				+	+		−	+	−	+
23	45	+				+	+		−	+	−	−
24	111	+				?	+		+	−	−	−
25	37	+				+	+		−	−	−	+
26	7	+				+	+		−	?	−	+
27	92	+				+		+	+	−	+	+
28	18, 26	+				+		+	−	+	−	+
29	75	+				+		+	+	+	−	−
30	4	+				+	?	?	−	+	−	+
31	41, 25, 22	+				+		+	−	+	−	−
32	30		+	+			+		+	+	−	+
33	19, 64		+		+		+		+	+	+	+
34	20, 59, 66, 67, 68, 71, 89, 103		+		+		+		+	−	+	+
35	58, 72, 95, 107		+		+		+		+	−	−	+
36	96, 97		+		+			+	+	−	+	+

TABLE 2—*Concluded*

GROUP	CULTURE NUMBER	LACTIC ACID		LIMITING UNDISSOCIATED LACTIC ACID			CO_2		GROWTH AT 20°C.	FERMENTATION		
		Inactive	Inactive + D	Below 0.050	0.051–0.130	0.131–0.200	Below 4 cc.	Above 4 cc.		Raffinose	Mannitol	Salicin
37	61, 105		+		+		?	?	+	−	+	+
38	14, 90, 94, 100, 102		+			+	+		+	−	+	+
39	8, 70		+			+		+	+	−	+	+
40	108		+			?		+	+	−	+	+
41	29	?	?	+			+		+	+	−	+
42	56, 57	?	?	+				+	−	+	−	−
43	52	?	?	+			?	?	+	+	+	−
44	54	?	?		+		?	?	−	+	−	+
45	17	?	?		+			+	−	+	−	−
46	51	?	?		+		?	?	−	+	−	−
47	110	?	?			+	?	?	+	−	−	−

could be safely placed on the basis of other characters. On this basis we have group A forming inactive acid only, and group B forming inactive plus dextro acid.

It was observed that there was a very obvious correlation between the type of lactic acid formed and the temperature limits within which the cultures grew. Of the 73 cultures forming only inactive acid, 58 (including 5 on which lactic acid was not determined but which were considered to belong with this group) or 79 per cent, did not grow at 20°C. On the other hand, of the 30 cultures which formed a mixture of inactive and dextro lactic all grew at 20°, and nearly all grew at 10°C. In other words, the cultures forming only inactive lactic acid had, as a rule, a narrow temperature range with few growing at 20° or lower while there was a 100 per cent positive correlation between the production of inactive plus dextro lactic acid and a wide temperature range extending always as low as 20° and frequently to 10°C. For the present we will include in the former group (A) only the 58 cultures forming inactive lactic and failing to grow at 20°C. The 16 cultures producing only inactive acid and growing at 20° are tentatively placed in a group designated C.

While it is generally admitted that the type of colony formed

on suitable agar is subject to some variation, the acidophilus colony is so characteristic that it is commonly used for identification. Our results as shown in table 3 and figure 6 indicate that this use is justified.

Almost without exception the cultures of the A group form X type colonies or a mixture of X and Y types. Fully 60 per cent

TABLE 3

Distribution of colony types

TYPE OF COLONY	GROUP A	GROUP B	GROUP C
	per cent	*per cent*	*per cent*
X	60.0	3.5	20.0
Mixture of X and Y	33.3	34.5	26.6
Y	6.6	62.0	53.3

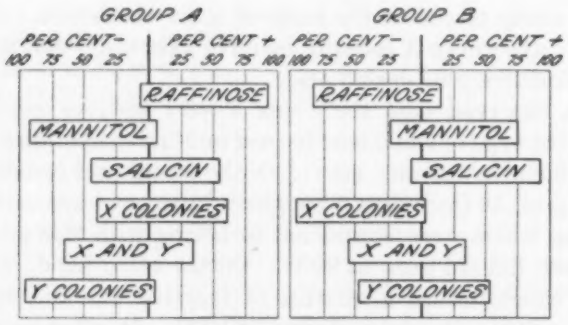


FIG. 6. CORRELATIONS WITH FERMENTATION REACTIONS AND COLONY TYPES

form X colonies. On the other hand, the colonies of the group B cultures are of the Y type or a mixture of Y and X with only 3.5 per cent forming X type alone.

The use of fermentation reactions without regard to proper correlation has tended to produce confusion rather than clarification in the classification of zymogenic bacteria but when these reactions are considered in their relation to other characteristics they become of real taxonomic significance. The fermentation

of raffinose, mannitol, and salicin is shown in table 4. In the fermentation of these substances the cultures of groups A and B exhibit distinct differences which are correlated with other characters. The A cultures, with very few exceptions, ferment raffinose and fail to ferment mannitol. About one-half ferment salicin. The B cultures quite uniformly fail to ferment raffinose but ferment mannitol and salicin. The correlation between type of lactic acid, temperature relations, colony type and these fermentations is too marked to be a coincidence.

It has been pointed out that the kinds of volatile acid are apparently identical for all of these cultures. The relation of the

TABLE 4

Fermentation of raffinose, mannitol, and salicin

SUGAR	GROUP A—INACTIVE ACID; NO GROWTH AT 20°C.				GROUP B—INACTIVE AND DEXTRO ACID; GROWTH AT 20°C.				GROUP C—INACTIVE ACID; GROWTH AT 20°C.			
	Number of cultures		Per cent of cultures		Number of cultures		Per cent of cultures		Number of cultures		Per cent of cultures	
	+	−	+	−	+	−	+	−	+	−	+	−
Raffinose	53	4	92.99	7.01	5	25	16.67	83.33	5	10	33.33	66.67
Mannitol	4	54	6.89	93.11	23	7	76.67	23.33	4	11	26.67	73.33
Salicin	32	26	55.17	44.83	28	2	93.33	6.67	8	7	53.33	46.67

volatile to total acid is also fairly uniform for the entire collection but figure 2 indicates that in general the cultures of group A produce a higher percentage of volatile acid than those of group B. While the cultures of both groups are distributed over the entire range, 83 per cent of the group B cultures show below 12 per cent of total acid volatile, while 80 per cent of the A cultures form more than 12 per cent volatile acids. It is not certain that the ratio of volatile to total acid is constant for any one culture but the distinct tendency of the A cultures to produce a higher proportion of volatile acid is of some significance.

The production of CO_2, shown in figure 2, differs from that of volatile acid in that the distribution of cultures shows two distinct modes. In the cultures forming less than 4 cc. of gas are included 80 per cent of the B cultures. The A cultures are more

variable but show a tendency to produce a distinctly greater quantity of CO_2. This agrees with Sherman's (1921) observation previously quoted. This property is very probably correlated with the formation of volatile acids and subject to the same variations.

The concentration of undissociated lactic acid at which growth ceases has never been used as a differentiating character, but it is well known that various species of streptococci stop growth at a definite hydrogen-ion concentration and that under uniform conditions this point is quite constant for each species. For each of our cultures there is a definite concentration of undissociated lactic acid at which growth stops and the cultures fall on this basis into three quite distinct groups.

Except for a few of the B cultures which will be discussed later, there is little evidence of a correlation of limiting undissociated lactic acid with other distinguishing characters. In figure 3 the A cultures are shown distributed in 3 modes made up of cultures of which one group was inhibited by a very low concentration, one by concentrations ranging from 0.060 to 0.120, and one by concentrations above 0.120. The B cultures are distributed through these 3 modes, but with 62 per cent in the group varying from 0.060 to 0.120. There is no apparent relationship between the ability to grow after a high concentration of undissociated lactic acid is reached and the activity of the culture in souring milk. In other words, a culture which curdles milk in a short time does not necessarily belong among the cultures continuing growth until the undissociated lactic acid reaches 0.120 or higher.

The concentration of phenol and indol in which these cultures will grow, although possibly not a determining factor, may be considered as an indication of their ability to maintain themselves in the intestinal tract. Figure 4 shows the distribution of the cultures in relation to their ability to grow in varying concentrations of phenol. Eighty-four per cent of the A cultures grew in concentrations as high as 1:250 to 1:400. On the other hand, none of the B cultures grew in concentrations as high as 1:350 and less than half were able to grow at 1:350 to 1:400. The difference

between the two groups is even more striking in their reaction to indol as shown in figure 5. Of the 41 A cultures on which this test was completed, 34 or 83 per cent grew in concentrations of from 1:1100 to 1:1900. Only one of the B cultures was able to grow at concentrations as high as 1:1900.

A group (C) of 15 cultures producing only inactive lactic acid has been set apart from the A group on the basis of their ability to grow at 20°C. or lower. The validity of a separation on this basis is doubtful. Some cultures grew at 20°, but not at 15°C. In others growth occurred at 20°C. in one test and failed on the second test. Some grew at temperatures as low as 10°C. There are too few cultures in this group to warrant a statistical treatment but some indication of their relationship to the cultures of group A may be found in the five charts. These data offer no suggestion of a basis for separationin to homogeneous groups. In the sugar fermentations the proportion of raffinose fermenters is smaller than would be expected. The colonies are largely of the smooth type. It is very probable that some at least of the C cultures should be included in the A group. If all were included the description of the A group would become somewhat less exact. It would then be necessary to state that while the typical culture does not grow at 20° a culture could not be excluded if it grew at 20°C. or lower. The percentage of raffinose fermenters would also be reduced and the percentage of mannitol fermenters increased. Finally, the occurrence of the Y type of colony in group A would be increased. It is probable that some of these cultures could be looked upon as atypical members of the A group while others could be completely separated by a more detailed study of a larger collection.

In the B group are 8 cultures which may possibly be separated from the group on the basis of their ability to carry the lactic acid concentration to a high point. While all other cultures are checked by a concentration of 0.120 moles of undissociated lactic acid per liter or lower, these continue growth until the concentration reaches 0.140 to 0.180. These cultures are almost identical in every respect and it is quite probable that in a larger collection

they could be differentiated as a separate species. We hesitate to do this on the basis of only 8 cultures.

The arrangement of these cultures according to source as shown in table 5 is enlightening. It will be noted that 73 per cent of the cultures known to be of intestinal origin fall in group A. However, a few cultures which were evidently considered by the laboratory in which they were isolated as typical *L. acidophilus* clearly belong in group B. It should be stated that of the 5 intestinal cultures in this group, 3 were not obtained from the laboratory in which they were originally isolated. Two were isolated from feces of rats in our own colony which had been fed for an extended period on glucose and lactose. The 9 Döderlein

TABLE 5

Distribution of cultures by sources

GROUP	VAGINA		INTESTINE		DENTAL		ACIDOPHILUS PREPARATIONS		UNKNOWN	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
A	6	66.7	17	73.9	8	33.3	5	38.5	22	64.7
B			5	21.7	14	58.3	2	15.4	9	26.5
C	3	33.3	1	4.4	2	8.3	6	46.1	3	8.8

cultures were all placed in groups A or C and it may be assumed that they were originally of intestinal origin.

Over half of the dental cultures belong in the B group. The 8 A group dental cultures which came from four laboratories conformed in all respects with the characteristics of the group. This is also true of the 14 dental cultures in the B group. In so far as the evidence from this collection indicates, the lactobacilli occurring in carious teeth are not of one species and are not usually of the acidophilus type.

On the assumption that group A is identical with *Lactobacillus acidophilus*, a description of this species may be formulated. For convenient comparison this is arranged in parallel columns with that of group B.

Group A (*Lactobacillus acidophilus.*)	*Group B* (Identity uncertain. Probably includes *L. bulgaricus* and possibly *L. casei.*)
Gram-positive rods; dimensions variable; frequently in chains; non-spore forming; non-motile.	Gram-positive rods; dimensions variable; frequently in chains; non-spore forming; non-motile.
Facultative anaerobe.	Facultative anaerobe.
Colonies on tomato-juice peptone agar have delicate filamentous outgrowths giving the colony a rough or woolly appearance. These may be mixed with smooth round colonies. In exceptional cases the colonies may all be smooth.	Colonies on tomato-juice peptone agar are round and smooth, but in some cultures these are mixed with colonies of the rough or woolly appearance. Only exceptional cultures give all rough colonies.
Optimum temperature is 37° to 40°C. The maximum growth temperature is usually 43°, but a few cultures grow at 46° and sometimes at 48°C. With possibly a few exceptions growth does not take place at 20°C.	Optimum temperature is 37° to 40°C. Upper limits of growth, 43° to 48° with occasional cultures growing at 50°C. All cultures grow at 15° and usually at 10°C.
Milk is curdled, in some cases very slowly, with a firm curd. Litmus, if present, is reduced.	Milk is curdled, in some cases very slowly, with a firm curd. Litmus, if present, is reduced.
In the fermentation of lactose a mixture of acids is formed which includes volatile acids usually in the proportion of between 12 and 20 per cent of the total acid. The volatile acids are formic, acetic, and butyric.	In the fermentation of lactose a mixture of acids is formed which includes volatile acids usually in the proportion of from 4 to 12 per cent of the total. The volatile acids are formic, acetic, and butyric.
The lactic acid produced is entirely of the inactive type.	The lactic acid produced is a mixture of the inactive and active. The active acid is dextro-rotatory.
Small amounts of CO_2 are formed in the fermentation of sugars. The amount is usually greater than that evolved by the cultures of the B group.	Small amounts of CO_2 are formed in the fermentation of sugars. This is usually less than is produced by the acidophilus cultures under the same conditions.
Maltose, sucrose, and raffinose are almost always fermented. Mannitol is rarely fermented, and about one-half the cultures ferment salicin.	Maltose and sucrose are almost always fermented. Only occasionally do cultures ferment raffinose. Mannitol and salicin are usually fermented.
Most of the cultures are resistant to phenol and in a suitable medium will grow in concentrations of 1:250 to 1:400.	Cultures are inhibited by phenol in concentrations of 1:400.
Cultures are usually tolerant of indol and will grow in concentrations of 1:1100 to 1:1900.	Cultures are sensitive to indol and rarely grow in concentrations greater than 1:1900.

SUMMARY

It was found possible to divide a collection of 103 lactobacilli into two distinct groups on the basis of kind of acid formed, temperature reactions, type of colony, fermentation of carbohydrates and inhibition by phenol and indol. One of these groups, including 58 cultures, agreed with the generally accepted concept of *Lactobacillus acidophilus* and showed a high positive correlation in the production of inactive lactic acid, the formation of rough or fuzzy colonies, failure to grow above 46° or as low as 20°C., fermentation of raffinose and failure to ferment mannitol. These cultures formed, as a rule, more CO_2, and a larger proportion of volatile acids, and grew in higher concentrations of phenol and indol than the members of the second group.

The cultures of the second group produced inactive and dextro lactic acid and grew at 20°C. or lower. The colony was usually of the smooth or Y type or a mixture of Y and X, rarely of the pure X type. Only occasionally did cultures ferment raffinose but mannitol and salicin were nearly always fermented. These strains were less active than the cultures of the first group in the formation of volatile acids and CO_2 and were inhibited by relatively dilute solutions of phenol and indol.

A third group, containing 15 cultures, differed from the first group in growing at 20°C. There was also sufficient variation in other characters to warrant setting them aside at least as atypical cultures.

All of the cultures produced some volatile acid consisting of a mixture of formic, acetic, and butyric.

A large proportion of the cultures of known intestinal origin were in the acidophilus group. A few of the dental cultures were of the acidophilus type, but the greater part belonged in the group producing inactive plus dextro lactic acid.

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STUDIES OF BACTERIUM COLI IN PRIVATELY OWNED RURAL WATER SUPPLIES[1]

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Received for publication October 3, 1932

The adequacy of the Standard Methods of Water Analysis (1925) procedure commonly in use in the United States for the bacteriological examination of water supplies, has been questioned by various investigators in the field of water bacteriology. New studies are being made constantly and the literature on the subject has accumulated rapidly in recent years. Excellent reviews and literature citations can be found in papers by Perry (1929), Ruchhoft, Kallas and Chinn (1931), Ruchhoft, Kallas, Chinn, and Coulter (1931), Poe (1931), and Prescott and Winslow (1931).

It is admittedly difficult to devise a method for determining the sanitary quality of drinking water which will be simple and rapid without being open to the criticism that it is not sufficiently comprehensive. Ruchhoft and his co-workers, and Poe, have mentioned some of the limitations of the Standard Methods procedure. Salle (1930), Dominick and Lauter (1929), Leahy (1930–1931), Noble (1928), and Tonney and Noble (1930–1931) have studied and suggested new methods to be added to, or to replace, bacteriological methods now in use for determining the sanitary quality of drinking water.

The study presented in this paper was undertaken to investigate the adequacy of the routine procedure of the Standard Methods of Water Analysis for bacteriological examination of water when applied to privately owned rural water supplies. The water samples received in our laboratory for examination are mostly from farms where the users are dependent on wells or

[1] Published as Contribution No. 145, Massachusetts Agricultural Experiment Station.

springs for their water supplies. An economic problem of great importance is involved because, if a supply is condemned, it is probable that the owner will be put to an embarrassing expense for reconstruction or for the location of a new source of supply. It occasionally happens that if the supply in use is condemned no other source is available.

SOURCE OF CULTURES

Two groups of strains of *Bact. coli* were studied in this investigation. Those of the first group were isolated from water samples received in the laboratory for routine bacteriological examination. All of these water samples were condemned as unfit for drinking purposes according to the presumptive, partially confirmed, and completed tests of the Standard Methods of Water Analysis. Eighty per cent of the samples were from shallow dug wells, 15 per cent from shallow driven wells and 5 per cent from springs. Ninety per cent of the shallow dug wells were curbed with loosely laid field stone. Loose boards or pieces of sheet metal were used as covers, and in no case were the covers elevated above the ground. Such construction makes it possible for surface water to gain easy access to the well.

The second group of *Bact. coli* strains were isolated from feces of members of the freshman class at the Massachusetts State College. All feces were from individuals who apparently were in normal health.

ISOLATION OF CULTURES

The water samples examined were collected in sterile bottles supplied by the laboratory. Where the samples came from a source sufficiently distant to require any considerable time in transit, containers were provided which permitted icing the samples. The longest time period between collection and examination of samples was thirty-six hours. The water samples were examined according to the procedure of the Standard Methods (1925) for the presumptive, partially confirmed, and completed tests.

The strains of *Bact. coli* selected for study were taken from colonies typical for the organism on Endo plates. Inoculations were made from these colonies into 0.5 per cent lactose broth (prepared according to Standard Methods). Attempts were made to purify the strains by streaking on Endo plates, but the method did not prove to be satisfactory. It seems probable that some of the failures with, and consequent criticism of, the methyl red, Voges-Proskauer, sodium citrate, and uric acid tests, by various investigators, may have been due to the employment of unpurified cultures of *Bact. coli*. For the present study the method of Ruchhoft, Kallas, Chinn and Coulter (1931) was chosen for purification of the cultures. The method is as follows: "Culture in tryptophane broth two to three hours and streak on E.M.B. Incubate plates for twenty to twenty-four hours. Repeat this process three times, each time fishing subcultures from all types of colonies on the E.M.B. plates. Then fish all subcultures to differential media, lactose broth and agar slants." The method was followed with the exception that Endo plates were used instead of E.M.B. For inoculation into the differential media twenty-four-hour-old cultures of purified strains on agar slants were employed.

As previously mentioned strains of *Bact. coli* of known fecal origin were isolated from fresh specimens of human feces. Precautions were taken in the collection of the feces to reduce outside contamination. Upon receipt of the feces in the laboratory the surface of each sample was seared with a hot spatula blade. Then a small portion was taken from the inside of each sample and put into sterile physiological saline. The saline was thoroughly shaken and a sterile wire loop was used to streak some of the resulting emulsion onto Endo plates. The plates were incubated for eighteen hours at 37°C., and well separated colonies were selected for inoculation into tryptophane broth. The strains isolated were purified by the method used for purifying the strains isolated from water. Twenty-four-hour-old cultures on agar slants were used for inoculating into the media employed for further study.

TESTS EMPLOYED

After the various strains from water and from feces had been isolated and confirmed as *Bact. coli* by the Standard Methods of Water Analysis procedure the methyl red, Voges-Proskauer, sodium citrate and uric acid tests were employed for separating the strains into the *Bact. coli*, or fecal group, and the *Bact. aerogenes*, or non-fecal group. The media were prepared and the tests carried out as recommended in the Standard Methods of Water Analysis, with the exception that in the Voges-Proskauer test acetyl-methyl-carbinol production was determined by a modification of the Standard Methods technic recommended by Werkman (1930).

In order to eliminate irregularities in the results obtained with the uric acid and sodium citrate media, the glassware employed was cleaned in cleaning solution and rinsed thoroughly in distilled water. The cleaning process was calculated to remove any traces of organic matter which might have furnished nutrient material for the bacteria. After washing, the glassware was dried quickly. A light inoculum into these media is necessary in order to avoid the transfer of nutrient material from the stock cultures into the differential media. There is also danger, if a heavy inoculum is used, of mistaking the resulting turbidity for bacterial growth.

Indol production has been widely used, especially by British workers (Levine, 1921) for the determination of "typical" *Bact. coli*. Levine found that among members of the colon group which were of intestinal origin, 91 per cent formed indol, while of those obtained from soil only 37.3 per cent formed indol. Since indol production has been widely used as an indicator of *Bact. coli*, the test is included in this study. The medium employed was 1 per cent tryptophane broth. Gore's (1921) plug test proved to be the most satisfactory indicator.

EXPERIMENTAL

Strains of *Bact. coli* were isolated from 172 samples of water which, as previously stated, had been examined in the laboratory

and condemned by the Standard Methods procedure as unfit for drinking purposes. A total of 223 strains were isolated and confirmed as *Bact. coli* by the presumptive test, the partially confirmed test and the completed test of the Standard Methods (1925) procedure. In addition to the strains isolated from water, 178 strains were isolated from feces. These strains were confirmed as *Bact. coli* by the Standard Methods procedure. All strains from water and from feces were studied for their reactions to the methyl red, Voges-Proskauer, sodium citrate, and uric acid tests, and for indol production. These tests were employed to differentiate the strains into *Bact. coli* of fecal type and non-fecal *Bact. aerogenes*.

The isolations from feces were made in order that the reactions of the strains from water might be compared with reactions of strains of known fecal origin. By careful purification of the strains employed, careful cleaning of glassware, and careful preparation of the media employed, inconsistent reactions of the strains in the different media were reduced to a minimum. One strain only, isolated from water, failed to give consistent results in duplicate cultures or in repeated experiments.

Three series of inoculations were made of all strains into the different media. In each series all cultures were made in duplicate. The reactions of the strains in the different media are shown in table 1.

DISCUSSION

Grouping of strains

The reactions in the differential media, shown in table 1, resulted in the grouping of the various strains studied into ten groups:

Group 1. *Bact. coli* of fecal type by all tests.

Group 2. *Bact. coli* of fecal type by the indol, methyl red, and Voges-Proskauer tests; non-fecal aerogenes by the sodium citrate and uric acid tests.

Group 3. This group is the same as group 2 except that the indol reaction is negative.

Group 4. Non-fecal *Bact. aerogenes* by all tests.

Group 5. Non-fecal *Bact. aerogenes* by all tests except the uric acid test; *Bact. coli* of fecal type by the uric acid test.

Group 6. Non-fecal *Bact. aerogenes* by all tests except the sodium citrate test; *Bact. coli* of fecal type by the sodium citrate test.

Groups 7, 8, 9, 10. A small number of strains giving irregular reactions.

TABLE 1

Reactions in different media of 223 strains isolated from water and 178 strains isolated from feces, all of which were confirmed as Bact. coli by the Standard Methods procedure

TEST	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6	GROUP 7	GROUP 8	GROUP 9	GROUP 10
Indol	+	+	−	−	−	−	−	+	+	−
Sodium citrate	−	+	+	+	+	−	−	−	+	−
Uric acid	−	+	+	+	−	+	+	+	−	−
Methyl red	+	+	+	−	−	−	+	−	−	+
Voges-Proskauer	−	−	−	+	+	+	−	+	+	−
Strains from water:										
Number of strains	135	45	19	8	7	4	2	1	1	1
Per cent of total	60.5	20.1	8.5	3.6	3.1	1.8	0.9	0.4	0.4	0.4
Strains from feces:										
Number of strains	166	3	2	4	1	0	0	0	0	2
Per cent of total	93.2	1.7	1.1	2.2	0.5	0	0	0	0	1.1

Agreement of reactions

In considering the agreement of the various tests in differentiating the strains studied into *Bact. coli* of fecal type and *Bact. aerogenes* of non-fecal type, the following statements can be made:

1. All media in agreement: Of the strains from water, 60.5 per cent, and of those from feces, 93.2 per cent, were *Bact. coli* of fecal type. Of strains from water, 3.6 per cent, and of those from feces, 2.2 per cent, were *Bact. aerogenes* of non-fecal type.

2. There was complete agreement between the methyl red and Voges-Proskauer tests.

3. The sodium citrate and uric acid tests failed to agree with each other with 15 of the strains from water and with 1 strain from feces. The sodium citrate test identified 7 of the strains

from water (3.1 per cent of the total) as *Bact. coli* of fecal type, and 8 strains (3.6 per cent of the total) as non-fecal *Bact. aerogenes*. The uric acid test reversed the identification. One strain from feces was identified as *Bact. aerogenes* by the sodium citrate test and as *Bact. coli* by the uric acid test.

4. Where the methyl red and Voges-Proskauer tests in agreement are compared with the sodium citrate and uric acid tests in agreement the following observation is made: There were 64 strains (28.7 per cent of the total) from water, and 5 strains (2.2 per cent of the total) from feces which were identified as *Bact. coli* of fecal type by the methyl red and Voges-Proskauer tests, and as non-fecal *Bact. aerogenes* by the uric acid and sodium citrate tests. Of 21 strains from water which were identified as non-fecal *Bact. aerogenes* by the methyl red and Voges-Proskauer tests, 8 were identified as *Bact. coli* of fecal type by the uric acid test, and 7 by the sodium citrate test. The 8 and 7 strains respectively were not duplicates in any instance.

Differential tests

The constancy of the indol test has been questioned, according to Levine (1921). However, in the present study the results of the indol reaction appeared to be as dependable as those of the other differential tests. Only 1 strain, isolated from water, and 2 strains, isolated from feces, which were identified as *Bact. coli* by all of the other differential tests in agreement failed to produce indol. In other groups of table 1, one or more of the other four differential tests employed agreed with the indol reaction.

The use of the sodium citrate and uric acid tests for differentiating *Bact. coli* of fecal type from *Bact. aerogenes* of non-fecal type was recommended by Koser (1918, 1924). He stated that where correlation did not exist between the methyl red and Voges-Proskauer tests the sodium citrate test served to assign the organism to its proper group and determine its source. He stated further (1924) that the reactions in sodium citrate and uric acid media correlated almost perfectly with the methyl red and Voges-Proskauer tests. Chen and Rettger (1920) reported that an almost perfect correlation could be established between

the methyl red, Voges-Proskauer, and uric acid tests. In the present study the uric acid and sodium citrate tests failed to agree with each other in a considerable number of instances, and they also failed to agree with the Voges-Proskauer and methyl red tests with a number of strains. A detailed statement of these failures in agreement are found in a previous section of this report.

In Bergey's manual (1930) the production of acetyl-methyl-carbinol (Voges-Proskauer reaction) by the *Bact. aerogenes* (*Aerobacter aerogenes*) group serves to distinguish them from the *Bact. coli* (*Escherichia coli*) group which are negative to the test. Chen and Rettger (1920) stated that when the Voges-Proskauer test is properly carried out it is constant in its results. Levine (1921) made a similar statement to the effect that "the reaction has been found to be very constant and it has proven satisfactory in the hands of practically all who have tried it." Levine states further that "the Voges-Proskauer reaction has been found very valuable and many investigators have observed that it is characteristic of the coli-like organisms of the soil and grain, while it is very rare to encounter intestinal members of the colon group which give this test." In the experiments reported in this paper only 5 out of 178 strains from feces were positive to the test, while 21 out of 223 strains from water were positive.

The methyl red test was devised by Clark and Lubs (1915). Chen and Rettger (1920) found the test useful but preferred the Voges-Proskauer test. Levine (1921) stated that the methyl red test correlates very well with the Voges-Proskauer reaction. In the present study the agreement between the two tests was perfect.

Analysis of results

In considering the significance of the results of the experiments reported in this paper it must be remembered that all of the strains chosen for study, from either water or feces, were characteristic for *Bact. coli* by the Standard Methods of Water Analysis procedure: All of the strains produced a sufficient quantity of gas in lactose broth, and on Endo's agar all gave a type of colony growth considered characteristic for *Bact. coli*. According to

the Standard Methods procedure these reactions are sufficient to justify condemning a water supply which yields a sufficient number of these organisms. In fact, all of the strains from water employed in study were from water samples which had been condemned by the Standard Methods procedure as unfit for drinking purposes. The condemnation of a privately owned rural water supply of the type commonly examined in this laboratory frequently involves financial outlay which the owner can ill afford. For that reason the present study was undertaken to determine how large a percentage of the water samples condemned by the Standard Methods procedure would have been condemned if methods commonly in use for differentiating the common gram-negative, lactose-fermenting organisms found in water samples into *Bact. coli* of fecal type and non-fecal *Bact. aerogenes* had been employed in addition to the Standard Methods routine for testing water.

From the results tabulated in table 1 the following observations were made:

1. All strains had been identified as *Bact. coli* by the Standard Methods procedure. The strains from water represented water samples which had been condemned by the Standard Methods procedure as unfit for drinking purposes.

2. If complete agreement among four differential tests, methyl red, Voges-Proskauer, sodium citrate and uric acid tests, were required for identification, 135 (60.5 per cent) of the strains isolated from water would be identified as *Bact. coli* of fecal type. The result would indicate that 39.5 per cent of the water samples represented had been condemned unfairly by the Standard Methods procedure. Of the strains isolated from feces 166 (93.2 per cent) would be identified as *Bact. coli* of fecal type.

3. If the methyl red and Voges-Proskauer tests were employed for identifying *Bact. coli* of fecal type, 202 (90.6 per cent) of the strains from water would be identified as *Bact. coli* of fecal type. The result would indicate that 9.4 per cent of the water samples represented had been unfairly condemned by the Standard Methods procedure. Of the strains from feces, 173 (97.2 per cent) would be identified as *Bact. coli* of fecal type. Since there

was complete agreement between the methyl red and Voges-Proskauer tests the figures would be the same for both.

4. If the sodium citrate test alone were employed to identify *Bact. coli* of fecal type, 143 (64.1 per cent) of the strains from water would be thus identified. The result would indicate that 35.9 per cent of the water samples represented had been condemned unfairly by the Standard Methods procedure. If the uric acid test alone were employed to identify *Bact. coli* of fecal type, 144 (64.5 per cent) of the strains from water would be thus identified; indicating that 34.5 per cent of the water samples represented had been condemned unfairly by the Standard Methods procedure. From the figures it would appear that there was close correlation between the sodium citrate and uric acid tests. Reference to table 1 will show, however, that 7 of the strains identified as *Bact. coli* by the sodium citrate test would be *Bact. aerogenes* by the uric acid test, and 8 of the strains identified as *Bact. coli* by the uric acid test would be *Bact. aerogenes* by the sodium citrate test. If agreement between the sodium citrate and uric acid tests were required to identify *Bact. coli* of fecal type, 136 (60.9 per cent) of the strains from water would be thus identified, indicating that 39.1 per cent of the water samples represented had been condemned unfairly by the Standard Methods procedure. Of the strains from feces, 168 (94.4 per cent) were identified as *Bact. coli* of fecal type by the sodium citrate test, and 169 (94.9 per cent) were thus identified by the uric acid test.

Reference to table 1 will show that there was a much more satisfactory agreement among the differential tests with the strains from feces than with the strains from water, and that a much higher percentage of the strains from feces than of the strains from water were identified as *Bact. coli* of fecal type. The question then arises as to the reason for the irregularity of the results with the strains from water, and whether the presence of the strains giving the irregular results should result in the condemnation of the water samples represented.

Certain investigators have stated that typical *Bact. coli* tend to disappear from water, leaving the non-fecal *Bact. aerogenes* and

related types predominant. Rogers (1918) found that non-fecal *Bact. aerogenes* has a distinctly greater viability in water than has *Bact. coli*. Winslow and Cohen (1918) reported similar results.

Since the disappearance of typical *Bact. coli* has thus been demonstrated it would seem to be reasonable to question the practice of condemning water samples because of the presence of certain members of the colon-aerogenes group which give variable results with differential tests. Such organisms might well be either indicators of a pollution so remote as to be of little danger to health, or perhaps be of non-fecal origin. It also appears reasonable to determine what reactions are regular and characteristic for *Bact. coli* of fecal type by studying strains which have been freshly isolated from feces. In the studies reported in this paper the strains from feces showed a much higher percentage giving uniform reactions characteristic of *Bact. coli* of fecal type in the differential media employed than was observed among the strains isolated from water.

The methyl red, Voges-Proskauer, sodium citrate and uric acid tests have been studied and found dependable by a sufficient number of reliable investigators to indicate that the evidence offered by these tests cannot be ignored. In the present study any or all of these tests showed that a considerable number of the strains from water, confirmed as *Bact. coli* by the Standard Methods procedure, did not give reactions characteristic of *Bact. coli* of fecal type when the differential tests were used. For that reason it seems certain that the Standard Methods procedure should be supplemented by one or more differential tests. The Standard Methods of Water Analysis (1925) tentatively recommends the methyl red, Voges-Proskauer, sodium citrate, and uric acid tests, with the statement that knowledge is not sufficiently complete to recommend any one test or group of tests.

One of the difficulties attending the water examination work as carried on in this laboratory is the impossibility of making sanitary surveys in any considerable number of cases. Entire reliance must be placed on the laboratory findings. Because of the economic problem involved it is not satisfactory to condemn

a water supply containing members of the colon-aerogenes group merely to be on the side of safety. It seems obvious that the Standard Methods procedure should be supplemented by differential tests to determine the character of the organisms present. However, the results of the differential tests employed in this study were so variable that the recommendation of any particular test or group of tests is not justified.

SUMMARY

1. Two hundred and twenty-three strains of the colon-aerogenes group isolated from water, and 178 strains isolated from feces, were studied. All strains were confirmed as *Bact. coli* by the Standard Methods procedure.

2. When the methyl red, Voges-Proskauer, sodium citrate, and uric acid tests were employed to differentiate the strains studied into *Bact. coli* of fecal type and non-fecal *Bact. aerogenes* it was observed that a considerably greater percentage of the strains from water than of the strains from feces gave irregular results.

3. The percentage of strains identified by the differential media as *Bact. coli* of fecal type was considerably greater among the strains isolated from feces than among those isolated from water.

4. The methyl red and Voges-Proskauer tests agreed perfectly with each other for all strains tested.

5. The sodium citrate and uric acid tests failed to agree with each other with 15 of the strains (6.7 per cent of the total) isolated from water. These tests failed to agree with each other with only one strain isolated from feces.

6. A considerably greater number of strains isolated from water were identified as *Bact. coli* of fecal type by the methyl red and Voges-Proskauer tests than by the sodium citrate and uric acid tests considered separately or together.

7. The results obtained in this investigation suggest that dependence on the Standard Methods procedure alone for determining the sanitary quality of drinking water, especially from privately owned supplies of unknown history, results in too many of the samples being condemned. The need of supple-

mentary differential tests is indicated, but results do not justify the recommendation of any particular test or group of tests.

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